

FORM PTO-1390 (Modified)  
(REV 11-98)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES

UAB-14402/22

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

CONCERNING A FILING UNDER 35 U.S.C. 371

097807501

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/US99/24148

15 October 1999

16 October 1998

TITLE OF INVENTION

POLYMORPHISM IN Fas PROMOTER AND Fas LIGAND PROMOTER

APPLICANT(S) FOR DO/EO/US

Robert P. KIMBERLY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)). *(unexecuted)*
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ Certificate of Mailing by Express Mail
20. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)

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PCT/US99/24148

UAB-14402/22

21. The following fees are submitted.

**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :**

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00
- ☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00
- ☒ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$100.00

Surcharge of \$130.00 for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).

☐ 20 ☐ 30

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	30 - 20 =	10	x \$18.00	\$180.00
Independent claims	11 - 3 =	8	x \$80.00	\$640.00
Multiple Dependent Claims (check if applicable)			<input type="checkbox"/>	\$0.00

**TOTAL OF ABOVE CALCULATIONS = \$920.00**

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).

☒

\$460.00

**SUBTOTAL = \$460.00**

Processing fee of \$130.00 for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).

☐ 20 ☐ 30

\$0.00

**TOTAL NATIONAL FEE = \$460.00**

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☒

\$40.00

**TOTAL FEES ENCLOSED = \$500.00**

Amount to be refunded	\$
charged	\$

☒ A check in the amount of **\$500.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. \_\_\_\_\_ in the amount of \_\_\_\_\_ to cover the above fees.

A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **07-1180** A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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39,204

REGISTRATION NUMBER

DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Robert P. Kimberly

Serial No.:

Filed:

For: POLYMORPHISM IN Fas PROMOTER AND Fas LIGAND PROMOTER

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**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to examination of the above-captioned application, please enter the following amendments and remarks.

**IN THE SPECIFICATION:**

On page 1, please add the following new sections after the title:

**Related Applications**

This application claims priority of PCT/US99/24148 filed 15 October 1999 claiming priority of U.S. Provisional Applications 60/139,659 filed 17 June 1999 and 60/104,644 filed 16 October 1998.

**Grant Reference**

The subject invention was made with government support from the National Institutes of Health (NIH RO1 4R33062). The government has certain rights in the invention.

**IN THE CLAIMS:**

Please cancel claims 22 and 23.

09/807501-000001

**REMARKS**

By the above amendments, original claims 22 and 23 have been canceled. Applicant respectfully requests entry of the above amendments.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with Markings to Show Changes Made."

Respectfully submitted,



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**VERSION WITH MARKINGS TO SHOW CHANGES MADE****IN THE SPECIFICATION:**

New sections entitled "Related Applications" and "Grant Reference" have been added on page 1 after the title.

**IN THE CLAIMS:**

Claims 22 and 23 have been canceled.

09607501.052701



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12Q 1/68</b>		<b>A1</b>	(11) International Publication Number: <b>WO 00/23623</b>
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(22) International Filing Date: 15 October 1999 (15.10.99)		<b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 60/104,644 16 October 1998 (16.10.98) US 60/139,659 17 June 1999 (17.06.99) US			
(71) Applicant (for all designated States except US): UAB RESEARCH FOUNDATION [US/US]; The University of Alabama at Birmingham, 1120-G Administration Building, 701 South 20th Street, Birmingham, AL 35294-0111 (US).			
(72) Inventor; and (75) Inventor/Applicant (for US only): KIMBERLY, Robert, P. [-/US]; 1900 University Boulevard, 429 THT, Birmingham, AL 35204-0006 (US).			
(74) Agents: COGEN, Ellen, S. et al.; Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C., Suite 400, 280 N. Old Woodward Avenue, Birmingham, MI 48009-5394 (US).			
(54) Title: POLYMORPHISM IN Fas PROMOTER AND Fas LIGAND PROMOTER			
(57) Abstract			

The use of a single nucleotide polymorph in a Fas ligand or Fas promoter region to diagnose disease is disclosed. The correlation of single nucleotide polymorphs in either a Fas ligand or Fas promoter region is correlated to individual susceptibility to autoimmune diseases or non-lymphatic cancer.

**POLYMORPHISM IN Fas PROMOTER AND  
Fas LIGAND PROMOTER**

**Field of the Invention**

5           The present invention relates generally to compositions and methods for identifying polymorphism in Fas promoter and Fas ligand promoters, and more particularly to identifying and typing single nucleotide polymorphisms in Fas promoter and Fas ligand promoter sequences which are independent indicators for disease susceptibility.

10                           **Background of the Invention**

          The proper function of the immune system of an organism is to bind and neutralize substances which are perceived to be foreign to that organism. T cells are a major component of the immune system and become activated to specific antigens. T cells function in binding substances displaying a particular antigen, as well as activating other components of the immune system cascade. 15           Immune system tolerance, and particularly T cell tolerance, of substances is maintained by activation induced cell death of substance specific T cells. Activation induced cell death of the T cells is mediated by Fas regulated apoptosis of the activated T cells that express Fas and Fas ligand. S. Nagata, 20           *Science* 1995, 267:1449-56. L.A. Norian et al., *Cutting Edge* 1998: 1079.

          CD95 is a type I transmembrane protein that belongs to the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor superfamily and has been mapped to the long arm of chromosome 10q23. I. Behrmann et al., *Eur. J. Immunol.* 1994, 24:3057-3062. Fas is expressed on the surface of many cell 25           types such as lymphocytes, epithelial cells, fibroblasts and a sub-population of

endothelial cells (F. Leithauser et al., *Lab. Invest.* 1993, 69:415-429), and upon Fas Ligand (FasL) engagement initiates a signal transduction cascade that results in programmed cell death (apoptosis).

The Fas ligand is also active in the creation of immune privilege sites and preventing graft rejection by inducing apoptosis in T cells. M. Li-Weber et al., *Eur. J. Immunol.* 1998, 28:2373-2383. Activation-induced cell death (AICD) is a major mechanism to maintain immune homeostasis. AICD occurs in mature T lymphocytes to limit antigen-specific response. Upon clearing antigens and/or pathogens from the host, activated T cells are deleted via the activation of apoptosis which depends on FasL expressing and the ligation of Fas. T. Brunner et al., *Nature* 1995, 373(6513):441-4; S.T. Ju et al., *Nature* 1995, 373(6513):444-8. Autocrine interaction of Fas and FasL leads to apoptosis of activated T cells which is critical for the maintenance of peripheral T cell tolerance. Paracrine interaction of Fas and FasL also play an important role. It has been reported that FasL expressing APCs can induce the depletion of responding T cells in the peripheral lymphoid organs, leading to systemic T cell tolerance that is specific for the antigens presented by APCs. FasL expressing APCs induce an earlier and more profound clonal deletion of antigen reactive T cells than does activation-induced suicide of the T cells (H.G. Zhang et al., *J. Immunol.* 1999, 162(3):1423-30). The regulation of FasL expressing is not only important in T cell induced tolerance, but also in other APCs such as B cell and activated macrophage induced clonal energy. Therefore, the inducibility and level of expression of FasL both under basal and



induction conditions could be very crucial in the process of maintaining immune tolerance and homeostasis.

Although the exact role of CD95 (Apo-1/Fas) in immune regulation has not been defined, several studies have implicated Fas as an important factor in the predisposition to disease. T. Zhou et al., *Nature Medicine* 1999, 5:42-48; T. Brunner et al., *Nature Medicine* 1999, 5:19-20. Reports have suggested an increase in Fas expression on T-cells of HIV infected children (C.B. Baumler et al., *Blood* 1996, 88:1741-1746), in blood cell cancers such as Hodgkin's disease and anaplastic large cell lymphomas (L. Xerri et al., *Histopathology* 1995, 27:235-241), and in SLE (W. Emlen et al., *J. Immunol.* 1994, 152:3685-3692; Y. Amasaki et al., *Clin. Exper. Immunol.* 1995, 99:245-250; E. Mysler et al., *J. Clin. Invest.* 1994, 93:1029-1034). The identification of polymorphisms in the promoter of CD95 allows for the study of genetic predisposition in these diseases.

Fas and FasL each play a critical role in the development and maintenance of the acquired immune repertoire. Activated lymphocytes express high levels of Fas which is involved in peripheral deletion of antigen primed lymphocytes and autoreactive T and B cells. S. Nagata, *Cell* 1997, 88:355-365. Studies using Fas and/or FasL deficient or defective mice have demonstrated that such mice develop either a lymphoproliferative syndrome (*lpr*) or a generalized lymphadenopathy (*gld*) along with systemic autoimmunity. P.L. Cohen et al., *Annu. Rev. Immunol.* 1991, 9:243-269. In humans, mutations in Fas protein lead to the development of human lymphoproliferative syndrome (ALPS). G.H. Fisher et al., *Cell* 1995, 81:935-

946; F. Rieux-Laucat et al., *Science* 1995, 268:1347-1349; J. Drappa et al., *N. Engl. J. Med.* 1996, 335:1643-1649. This CD95 defect is characterized by hypercellularity of secondary lymphoid tissues. However, only rare mutations in Fas have been found in SLE.

- 5           FasL is type II membrane protein and a member of the TNF superfamily expressed mainly on activated T lymphocytes. The extracellular region of FasL binds to CD95 (Fas) and the binding induces apoptosis in activated cells expressing Fas. FasL and Fas interaction plays very important roles in maintaining the immune privilege sites (T.S. Griffith et al., *Science* 1995, 270(5239):1189-92), and in immune evasion by cancer cells (S. Nagata, *Nature Medicine* 1996, 2(12):1306-7), and in peripheral immune tolerance (S. Nagata et al., *Science* 1995, 267(5203):1449-56). FasL promotes immune tolerance through the induction of apoptosis and deletion of activated T, B lymphocytes and macrophages. G.C. Singer et al., *Immunity* 1994, 1(5):365-371; K.B. Elkon et al., *Current Opinion in Immunology* 1996, 8(6):852-9; D. Ashany et al., *Proc. Natl. Acad. Sci. U.S.A.* 1995, 92(24):11225-9. Deletion through apoptosis of activated cells in the immune system presumably eliminates potentially autoreactive T and B lymphocytes. In addition, it has been found that cells in immunologically privileged sites such as Scitoli's cells of testis and parenchymal cells of the anterior chamber of the eye, express FasL. Any activated T cell bearing Fas that enters such a site would encounter cells expressing FasL and receive a death signal, thereby preventing an immune response. T.S. Griffith et al., *Science* 1995, 270(5239):1189-92. The critical role of the FasL and Fas interaction in the maintenance of immune

tolerance and prevention of autoimmune disease has been demonstrated by the finding that mutations of Fas or FasL genes lead to autoimmune disease in lpr/lpr and gld/gld mice, respectively. R. Watanabe-Fukunaga et al., *Nature* 1992, 356(6367):314-7; T. Takahashi et al., *Cell* 1994, 76(6):969-76. Human autoimmune lymphoproliferative syndrome (ALPS or Canale-Smith Syndrome) characterized by defective lymphocyte apoptosis, lymphocyte accumulation, and humoral autoimmunity has been found to be associated with inherited mutations in the Fas. J. Drappa et al., *N. Engl. J. Med.* 1996, 335(22):1643-9; G.H. Fisher et al., *Cell* 1995, 81(6):935-46; F. Rieux-Laucat et al., *Science* 1995, 268(5215):1347-9; M.C. Sneller et al., *Blood* 1997, 89(4):1341-8; M.C. Sneller et al., *J. Clin. Invest.* 1992, 90(2):334-41. FasL gene mutation has also been implicated with SLE in one patient. J. Wu et al., *J. Clin. Invest.* 1996, 98(5):1107-13. These findings underscore the importance of Fas and FasL interaction in the maintenance of immune tolerance and prevention of autoimmune disease in humans.

The genomic organization of Fas and the characterization of the promoter was described by Cheng and colleagues. J. Cheng et al., *J. Immunol.* 1995, 154:1239-1245. These studies demonstrated that the promoter is organized into a basal promoter with both enhancer and silencer regions. F. Rudert et al., *Cell Biology* 1995, 14:931-937.

The role of the Fas ligand is currently not fully understood. Nonetheless, Fas ligand does serve as a feedback control on T cell response to foreign substances to the organism.

Autoimmune disease is often characterized by abnormal deposits of immune complexes. Such deposits may result from excessive immune complex formation, inadequate antibody production, Fas ligand deficiencies, or Fas receptor anomalies. Receptors that are incapable of binding and/or releasing Fas ligand would yield inadequate suppression of T cell activity thereby resulting in immune complex deposition.

Such abnormal deposits are associated with a variety of diseases including several types of human glomerulonephritis and autoimmune lymphoproliferative syndrome defects in Fas (CD95) mediated apoptosis. In particular, systemic lupus erythematosus (SLE) is associated with immune complex deposits.

SLE is considered a prototypic systemic autoimmune disease with involvement of multiple organ systems and extremely diverse clinical manifestation. Considerable evidence suggests that the development of SLE appears have a strong genetic basis. Since disease appears to involve the emergence and activation of both autoreactive T cells and B cells, it seems likely that susceptibility genes will influence this breakdown of immune tolerance in either or both lymphocyte population. It is commonly believed that the interactions of multiple genes and environmental factors result in the susceptibility to SLE. SLE is thought to represent a failure of the regulatory mechanisms of the immune system. Recent data from different sources suggest that genes on chromosome 1 are likely to play an important role in SLE. B.P. Tsao et al., *J. Clin. Invest.* 1997, 99(4):725-31; P.M. Gaffney et al., *Proc. Natl. Acad. Sci. U.S.A.* 1998, 95(25):14875-9; K.L. Moser et al., *Proc. Natl. Acad.*

Sci. U.S.A. 1998, 95(25):14869-74; R. Shai et al., *Hum. Mol. Genet.* 1999, 8(4):639-44. The human FasL gene consists of four exons and spans about 8 kb on chromosome 1q23 (T. Takahashi et al., *International Immunology* 1994, 6(10):1567-74). This region is also syntenic with the murine lupus susceptibility loci on chromosome 1q. T.J. Vyse et al., *Current Opinion in Immunology* 1996, 8(6):843-51. Polymorphisms in many genes, illustratively including CR1, FcγRIIA, FcγRIIIA, PARP, encoding molecules with relevant immunological functions or DNA repair on chromosome 1q have been associated with SLE. P. Cornillet et al., *Clinical & Experimental Immunology* 1992, 89(1):22-5; J.E. Salmon et al., *J. Clin. Invest.* 1996, 97(5):1348-54; J. Wu et al., *J. Clin. Invest.* 1997, 100(5):1059-70; B.P. Tsao et al., *J. Clin. Invest.* 1999, 103(8):1135-40.

Suppression of the immune response is desirable in such instances of autoimmune disease and following transplantation. In autoimmune diseases, the host immune system incorrectly recognizes body tissue as being foreign with the resulting activation of an immune response towards the body tissue. If a predisposition to an autoimmune disease could be detected, then early and aggressive therapies can be initiated to slow or prevent an autoimmune response. Treatments might include lifestyle modification, chemical therapeutics and gene therapies. Likewise, transplant recipients' medication dosage to deter rejection of the transplant depends on the levels of Fas ligand expression. It would therefore be advantageous if a marker suggestive of predisposition to an autoimmune disease existed along with a method for identifying such a marker.

It has recently been demonstrated that FasL is expressed in human melanoma, hepatocellular carcinoma, lung cancer, astrocytoma, esophageal carcinoma, gastric adenocarcinomas, ovarian carcinoma, and colon adenocarcinomas. m. Hayne et al., *Science*, 1996, 274(5291):1363-6; S. Strand et al., *Nat. Med.*, 1996, 2(12):1361-6; G.A. Niehans et al., *Cancer Res.* 1997, 57(6):1007-12; P. Saas et al., *J. Clin. Invest.* 1997, 99(6):1173-8; M.W. Bennett et al., *J. Immunol* 1998, 160(11):5669-75; M.W. Bennett et al., *Gut* 1999, 44(2):156-62; H.Rabinowich et al., *J. Clin. Invest* 1998, 101(11):2579-88; J. O'Connell et al., *J. Exp. Med.* 1996, 184(3):1075-82; J. O'Connell et al., *J. Pathol.* 1998, 186(3):240-6; K. Shiraki et al., *Proc. Natl. Acad. Sci. U.S.A.* 1997, 94(12):6420-5. Recent evidence suggests that tumor-infiltrating lymphocytes (TILs) are susceptible to Fas-mediated counterattack.

In esophageal cancer, the extent of apoptosis of TILs was found to vary regionally within the tumors in relation to the local status of FasL expression, local expression of FasL by nests of tumor cells was associated with apoptotic depletion of TILs. M.W. Bennett et al., *J. Immunol* 1998, 160(11):5669-75. In addition to local defense, FasL expression facilitate the establishment of tumor metastases in tissues such as the liver, where the indigenous normal cells are themselves sensitive to FasL. K. Shiraki et al., *Proc. Natl. Acad. Sci. U.S.A.* 1997, 94(12):6420-5. Significant correlation between tumorigenicity and expression of FasL has raised the possibility that FasL may be a useful diagnostic marker for malignant melanomas. A. Maeda et al., *Br. J. Dermatol.* 1998, 139(2):198-206.

Despite improved understanding of the important physiological roles for FasL in immune-homeostasis and immune privileged sites and cancer immunity, little is yet known about the regulation of FasL expression in these cell types. It has been reported that NF- $\kappa$ B, NF-AT, Egr-3, and several unknown regulatory elements in the FasL promoter are important in the regulation of FasL expression in Jurkat T cells. K.M. Latinis et al., *J. Biol. Chem.* 1997, 272(50):31427-34; M. Li-Weber et al., *Eur. J. Immunol* 1998, 28:2373-2383; L.A. Norian, *J. Immunol* 1998, 161(3):1078-82; Holtz-Heppelmann et al., *J. Biol. Chem.* 1998, 273(8):4416-4423; P.R. Mittelstadt et al., *Mol Cell Biol* 1998, 18(7):3744-51. There are little information about the natural genetic variations of FasL promoter region that may affect the FasL expression both in T cells and cancer cells.

It has recently been established that colon cancers typically express FasL, a potent mediator of immune privilege. Expression of FasL potentially enables colon tumors to counterattack Fas-sensitive anti-tumor immune effector cells by delivering a Fas-mediated apoptotic death signal. J. O'Connell et al., *J. Pathol.* 1998, 186(3):240-6.

The liver is the most common site for development of metastatic colorectal cancer. The remarkably high incidence of liver metastases in patients with colorectal cancer suggests that the liver provides an environment conducive to the development of metastasis. It has been demonstrated that FasL expressing colon cancer cells promoted local tumor growth by inducing apoptotic cell death in normal hepatocytes at the tumor margin in colorectal hepatic metastasis (K.F. Yoong et al., *Am. J. Pathol.* 1999, 154(3):693-703).

There are significant correlation between tumorigenicity and expression of FasL for melanomas, and it is proposed that FasL may be a useful diagnostic marker for malignant melanomas. W.M. Mulder et al., *Gut* 1997, 40(1):113-9; R.Kiessling et al., *Gut* 1997, 40(1):153-4.

- 5           The present invention utilizes four polymorphic sites within one kb of FasL promoter region. FasL promoter is highly polymorphic and these polymorphisms are operative in the regulation of FasL expression in cancer cells. Furthermore, three polymorphisms that are sitting in different transcription factor binding sites have been characterized herein, as well as two
- 10   TCF/LEF1 elements in the FasL promoter that are operative in the FasL expression in cancer cells.

- TCF is a potent architectural factor which facilitates assembly of multiprotein enhancer complexes. TCF selects a subset of target genes by binding to these co-operatively with other cell-type-specific factors, thus
- 15   programming their transcriptional competence to respond to *Wnt* signaling. While earmarking these genes, TCF keeps them in a repressed state, presumably with the help of a co-repressor. On *Wnt* signaling,  $\beta$ -catenin translocates into the nucleus and binds to TCF, thereby break up the repressive complexes on TCF earmarked genes. In addition to relieving repression  $\beta$ -
- 20   catenin serves as transcription activator and increase the rate of transcription. Therefore, it is possible that these two TCF binding elements (TBE1 and TBE2) are very important in the regulation of FasL expression and responsible for the tight control of FasL expression in leukocytes (manuscript in preparation). Mutation of the adenomatous polyposis coli (APC) tumor



suppressor gene initiates the majority of colorectal cancers. One consequence of this inactivation is constitutive activation of  $\beta$ -catenin/TCF-mediated transcription. V.Korinek et al., *Science* 1997, 275(5307):1784-7; P.J. Morin et al., *Science* 1997, 275(5307):1787-90; B. Rubinfeld et al., *Science* 1997, 275(5307):1790-2; A.B. Sparks et al., *Cancer Res.* 1998, 58(6):1130-4.

In accord with the present invention there is provided a composition, a method, and a diagnostic whereby predisposition to an autoimmune disease, infectious disease, transplant rejection or immune system resistant tumor growth is identified through nucleotide typing the Fas promoter and/or the Fas ligand promoter region of an organism for single nucleotide polymorphs existent therein.

#### Summary of the Invention

The present invention relates to the use of nucleic acid to diagnose disease, and in particular the use of nucleic acid from cells to diagnose autoimmune disease or cancer. The present invention correlates the susceptibility of an individual to a variety of autoimmune diseases and non-lymphatic cancers through typing nucleotide sequences of Fas promoter and Fas ligand promoter. A diagnostic method for determining autoimmune disease or cancer susceptibility according to the present invention includes genotyping a subject in a Fas ligand or Fas promoter region. A further method for identifying susceptibility to a disease includes identifying a first either Fas or Fas ligand promoter genotype at a nucleotide site in a host and quantifying susceptibility of the host to the disease. Thereafter, susceptibility of the host to the disease is compared to susceptibility of a second host having a second

either Fas or Fas ligand promoter genotype, the second genotype being dissimilar from the first genotype. A Fas ligand or Fas promoter single nucleotide polymorphism is correlated with disease susceptibility.

5 A diagnostic test kit for disease susceptibility according to the present invention includes reagents for assaying for a single nucleotide polymorph within a Fas or Fas ligand promoter gene of a host together with instructions for the use thereof as a diagnostic.

The present invention further includes the use of a single nucleotide polymorph within either a Fas ligand or Fas promoter gene of an individual for  
10 determining susceptibility of said host to a disease.

#### **Brief Description of the Drawing**

Figure 1 shows nucleotide polymorphisms in FasL promoter region in the one kb FasL promoter region. A) Polymorphism at nucleotide position -844 in the FasL promoter region. Sequence alignment of a portion of FasL  
15 promoter from three different donors is shown. Donors homozygous for nt -844T (upper panel), homozygous for nt -844C (lower panel), and heterozygous for nt -844T/C (middle panel) are shown. B) Polymorphism at nucleotide position -756 in the FasL promoter region. Sequence alignment of a portion of FasL promoter from three different donors is shown. Donors  
20 homozygous for nt -756A (upper panel), homozygous for nt -756G (lower panel), and heterozygous for nt -756A/G (middle panel) are shown. C) Polymorphism at nucleotide position -478 in the FasL promoter region. Sequence alignment of a portion of FasL promoter from three different donors is shown. Donors homozygous for nt -478A (upper panel), homozygous for nt

-478T (lower panel), and heterozygous for nt -478A/T (middle panel) are shown. D) Polymorphism at nucleotide position -205 in the FasL promoter region. Sequence alignment of a portion of FasL promoter from three different donors is shown. Donors homozygous for nt -205C (upper panel),  
5 homozygous for nt -205G (lower panel), and heterozygous for nt -205C/G (middle panel) are shown.

Figure 2A shows a sequence analysis of the FasL promoter and the description of oligonucleotides used in electrophoretic mobility shift assay (EMSA) experiments. The C/EPB $\beta$  element has been located between position  
10 -848 and -839. The underlined polymorphic site at position -844 is within the core of C/EBP binding motif. The sequences of the sense strand, in the 5' to 3' orientation, of various double-stranded oligonucleotides used for EMSA have been aligned to the genomic sequence for comparison. The potential binding sequences are included in the rectangular squares.

15 Figure 2B shows EMSA targeting the C/EPB $\beta$  binding element with radioactive labeled double strand -844C (lane 1-5) and -844T oligos (lane 6) which were incubated for 15 min. with 8  $\mu$ g of Jurkat cell nuclear extract. Competition experiments are performed by preincubating with 100 fold molar excess of the unlabeled C/EBP consensus oligonucleotides (lane 1), or  
20 unlabeled specific oligos (lane 3), or non-specific oligos (lane 5) for 20 min. at room temperature. Supershift experiments are carried out by preincubating with anti-C/EBP $\beta$  antibody for 30 min. at room temperature (lane 4). The arrows and bracket indicate the position of specific complex.

Figure 2C shows FasL promoter activity of different genotypes of C/EBP $\beta$  in colon cancer cell line SW480. SW480 cells are transiently transfected with one kb FasL promoter pGL3-Basic reporter constructs containing either -844C or -844T alleles (two constructs are different at position -844 only). The RLU is given as the means  $\pm$  SEM. The star symbol indicates that there are significant expression differences between two genotypes ( $p < 0.01$ ).

Figure 3 shows sequence analysis of the FasL promoter and the description of oligonucleotides used in EMSA experiment. The 1065 nucleotide long FasL promoter region was amplified from position -1032 to +33 with the sense primer and anti-sense primer. The C/EBP $\beta$  element has been located between position -848 and -839. The underlined polymorphic site at position -844 is within the core of C/EBP binding motive. The sequences of the sense strand, in the 5' to 3' orientation, of various oligonucleotides used for EMSA have been aligned to the genomic sequence for comparison. The potential binding sequences are included in the rectangular squares.

Figure 4A shows the sequence analysis of the FasL promoter and the description of oligonucleotides used in EMSA experiments. The underlined polymorphic site at position -756 is within the unknown transcription factor binding motif. The sequences of the sense strand, in the 5' to 3' orientation, of various double-stranded oligonucleotides used for EMSA have been aligned to the genomic sequence for comparison.

Figure 4B shows EMSA of SW480 and Jurkat cell line binding of radiolabeled probe containing position -756 of FasL promoter region.

Figure 4C shows FasL promoter activity of different genotypes at nucleotide position -756 of pGL3-Basic constructs in colon cancer cell line SW480 (two constructs are different at position -756 only). Nucleotide substitution at -756 significantly affected the 1 kb FasL promoter activity in the colon cancer cell line SW480 with wild type (-756A) has more than double activity as the mutant (-756G). The RLU is given as the means  $\pm$ SEM. The star symbol indicates that there are significant differences compared to wild type constructs (WT) ( $P < 0.01$ ).

Figure 5A shows the location of putative proximal TCF/LEF-1 binding element in the FasL promoter region. The putative proximal TCF/LEF-1 binding element (TBE2) of FasL promoter has been identified between position -206 and -200 with which the underlined polymorphic nucleotide position (-205C/G) is found. The sequences of the sense strand, in the 5' to 3' orientation, of various double-stranded oligonucleotides used for EMSA have been aligned to the genomic sequence for comparison. The binding sequences are included in the rectangular squares.

Figure 5B shows EMSA of SW480 cell line binding of radiolabeled probe containing putative TCF/LEF-1 corresponding to the -205 polymorphism of FasL promoter.

Figure 5C shows the location of putative distal TCF/LEF-1 binding element in the FasL promoter region. The putative distal TCF/LEF-1 element (TBE1) has been located between position -838 and -832. The underlined nucleotides indicate the induced mutation sites within TCF/LEF-1 binding motif on EMSA oligos. The sequences of the sense strand, in the 5' to 3'

orientation, of various double-stranded oligonucleotides used for EMSA have been aligned to the genomic sequence for comparison. The binding sequences are included in the rectangular squares.

Figure 5D shows EMSA of SW480 cell line binding of radiolabeled probe containing putative TCF/LEF-1 corresponding to the nucleotide position -838 and -832.

Figure 6A shows a map of the FasL promoter, indicating 1026 bp region containing two TCF/LEF-1 binding elements (TBEs). The 1026 bp FasL promoter fragment is engineered to contain mutations in either distal (TBE1M/2W), proximal (TBE1W/2M), or both distal and proximal (TBE1M/2M), and each fragment is placed upstream of pGL3-Basic vector to obtain reporter constructs. Three copies of TBE2 were placed in front of SV40 promoter in pGL3-Promoter vector to examine enhancer activity in colon cancer cells.

Figure 6B shows enhancer activity of proximal TCF/LEF-1 binding element (TBE2) of FasL promoter in colon cancer cell lines SW620 and SW480. Three copies of 18 bp fragment containing TBE2 wild type (3XTBE2W) or TBE2 mutant (3XTBE2M) are constructed in front of a basal SV40 promoter within the pGL3-Promoter vector. The TBE2 multimer pGL3-Promoter constructs are transfected into colon cancer cell lines SW620 and SW480 with pGL3-Promoter vector DNA as a control. Multimer TBE2 enhanced the activity of SV40 promoter in both SW620 and SW480 cells and the wild type (TBE2W) is a better enhancer than the mutant (TBE2M). The

RLU is given as the means  $\pm$ SEM. The star symbol indicates that there are significant differences between 3XTBE2W and 3XTBEM ( $P < 0.001$ ).

Figure 6C shows FasL promoter activity of different TCF/LEF-1 mutant constructs in colon cancer cell line SW480. Mutation of either distal TBE (TBE1M/2W) or proximal TBE (TBE1W/2M) did not significantly affect the 1 kb FasL promoter activity compared to the wild type in the colon cancer cell line SW480 ( $P = 0.50$  for TBE1W2M and  $P = 0.30$  for TBE1M/2M). However, simultaneous mutations of both distal and proximal TCF/LEF-1 binding elements decrease FasL promoter activity in the colon cancer cell line SW480 ( $P < 0.01$ ). The RLU is given as the means  $\pm$ SEM. The star symbol indicates that there are significant differences compared to wild type constructs (WT).

Figure 7 shows A) The polymorphic nucleotide positions detected on FasL promoter region and their respective nucleotide substitutions. The 1065 nucleotide long FasL promoter region was amplified from position -1032 to +33 with the sense primer and anti-sense primer. B) Haplotypes deduced from homozygous donors and TA cloning of several heterozygous donors and their designated names used in the experiments. The first two haplotypes (-844C and -844T) are the most common haplotypes in populations. The mut3 haplotypes are more common in African American population than in North American Caucasians. In contrast, the frequency of DM844T478T haplotype is more common in Caucasians than in African Americans. The frequencies of the last two haplotypes are least common haplotypes.

Figure 7C shows FasL promoter activity of different haplotype constructs in colon cancer cell line SW480. Double changes of either C/EBP $\beta$  and -205 (DM844T205G) or at C/EBP $\beta$  and -756 (DM844T756G) did not significantly affect the 1 kb FasL promoter activity in the colon cancer cell line SW480 (P=0.06476 for DM844T205G and P=0.1937 for DM844T756G). However, simultaneous mutations of three sites with -844T, -756G, and -205G (MUT3 haplotype) can significantly decrease FasL promoter activity in the colon cancer cell line SW480 (P<0.05). Data are representative of four independent experiments. The RLU is given as the means  $\pm$ SEM. The star symbol indicates that there are significant differences compared to wild type constructs (WT).

Figure 8 shows an EMSA gel showing effects of single nucleotide polymorphisms within the 5' Fas ligand promoter region at -844 (A and A'), -756 (B and B') and -205 (C and C').

Figure 9 is a novel polymorphism in the Fas promoter at nucleotide -690. Sequencing is performed using the 945 bp PCR product using Big Dye Terminator Chemistry (PE Applied Biosystems, Foster City, CA). The reaction is run and analyzed using an ABI 377 automated sequencer (PE Applied Biosystems). Sequencing is performed in both the forward and reverse direction over the entire 945 bp region using a series of primers outlined in Figure 8 on 30 SLE patients and 36 normal donors. A new polymorphism is identified at nt -690 of the Fas promoter which is shown above. (A) The ABI file tracing of a TT homozygote. (B) The ABI 377 file tracing of a T/C heterozygote.



Figure 10 is a schematic representation of the Fas promoter with the location of PCR and sequencing primers. PCR amplification of genomic DNA from 30 ethnically diverse SLE patients and 36 ethnically diverse normal donors is performed using the primers at nt -914 and +31. The resulting PCR product is subject to separation on a 3% agarose gel. The 945 bp product is purified from the agarose gel using a QIAQuick Gel Extraction Kit (QIAGEN, Valencia, CA). Shown above is a schematic diagram of primer sequences and approximate locations that were used for PCR and subsequent sequencing.

Figure 11 is a polymorphism at nucleotide -670. Sequencing is performed on the 945 bp PCR product of the distal Fas promoter using Big Dye Terminator Chemistry (PE Applied Biosystems). The subsequent reaction is run and analyzed using an ABI 377 automated sequencer (PE Applied Biosystems). Sequencing is performed in both the forward and reverse directions over the entire 945 bp PCR product using a series of primers outlined in Figure 8. Sequencing is performed on 30 SLE patients and 36 normal donors. The polymorphism found at nt -670 was previously reported by Huang and colleagues. Q.I.R. Huang et al., *Molecular Immunology* 1997, 34:577-582. (A) The ABI 377 file tracing of an A/A homozygote. (B) The ABI 377 file tracing of a A/G heterozygote. (C) The ABI 377 file tracing of a G/G homozygote.

#### **Detailed Description of the Invention**

The present invention relates to compositions and methods for identifying single nucleotide polymorphisms in the Fas promoter and/or Fas ligand promoter regions which correspond to disease associations. These

5 disease associations allow one to determine host susceptibility to, or severity of the disease. The single nucleotide allelic differences in Fas promoter and Fas ligand promoters may be determined by direct DNA sequencing or other conventional means. The haplotype of individuals when determinable from direct cloning or inference based on previous cloning work allows for linked combinations of single nucleotide polymorphs to be disease associated.

Polymorphs in the 5' promoter region of Fas (CD95) are not well characterized. Q.I.R. Huang et al., *Molecular Immunology* 1997, 34:577-582. The present invention confirms a A-G single nucleotide polymorphism (SNP) in the proximal promoter at nucleotide -670.

A direct sequencing approach is utilized to screen the proximal 5' promoter of CD95 from -914 to +30 of the coding region for single nucleotide polymorphisms. The present invention identifies a novel polymorphism at -690. The -690 site is located within the putative enhancer region of CD95. A higher representation of the -670G allele is seen in patients with SLE as compared with the normal control group. The new SNP identified at -690 does not associate with SLE.

TAAG and TGAG haplotypes of Fas ligand promoter at positions -844, -756, -478 and -205, respectively, are associated with individuals suffering from SLE in greater than 90% of a diagnosed disease group.

The present invention is based on the finding that single nucleotide allelic polymorphism in a Fas promoter or Fas ligand promoter correlates with autoimmune disease occurrence, tumor resistance to the immune system through FasL expression and infectious disease susceptibility. Due to the

important role of Fas and Fas ligand in feedback control of the immune response, the present invention has utility as a diagnostic for identifying high risk individuals that warrant early and aggressive treatment. As a diagnostic for infectious disease, the present invention has utility in predicting susceptibility to specific microbes thereby guiding the use of therapeutics. As a diagnostic for autoimmune disease, the present invention has utility in diseases illustratively including SLE, systemic vasculitis, autoimmune lymphoproliferative syndrome, glomerulonephritides, Sjogren's syndrome and IgA nephropathy. Identification of the appropriate allelic forms further allows for gene therapy transduction of host cells to correct hereditary limitations in an individual's Fas or Fas ligand promoter genes through delivery of an operative, insertable promoter gene or a promoter-Fas ligand translatable plasmid to a defective host cell. Generally, a predominant "normal" gene of the total human population or a derivative thereof would be delivered.

The present invention provides a method for identifying the Fas or Fas ligand promoter single nucleotide allelic pattern in individuals which involves testing DNA from individual patients for the presence of different allelic variants and haplotyping the individual for a series of SNPs suggestive of a particular disease. The present invention also encompasses the identification analysis of new single nucleotide allelic forms within Fas ligand promoter. Analysis being achieved using methods well known in the art, such as direct DNA sequencing; single strand conformational polymorphism analysis (SSCP); "HOT" cleavage; denaturing gradient gel electrophoresis (DVGE) and

combinations thereof. The various methods of the present invention are detailed in application 60/094,096 filed July 24, 1998.

Once a new polymorphism has been identified, immunological and/or molecular biological tests are used to genotype patients for the presence or absence of a given single nucleotide polymorphism. For example, monoclonal antibodies specific to the protein encoded by a newly identified single nucleotide allele are prepared by well known methods. These antibodies can be used for genotyping the patient population as described above. Alternatively, allele specific oligonucleotides may be designed for use as probes and/or as primers in hybridization or PCR based detection methods, respectively.

Through the establishment of statistically significant correlations between the different single nucleotide polymorphic allelic forms of Fas promoter or Fas ligand promoter and various physiological or clinical manifestations of variable Fas ligand function, the role of naturally occurring point mutations in specific autoimmune disease, predispositions to infectious disease, transplant rejection or treatment resistant tumor growth is identified in homozygous and heterozygous genotype donors and in stable transfectants. It is appreciated that preliminary studies of a specific condition are facilitated by studying homozygous individuals, no limitation on the present invention exists in regard to heterozygous genotypes. These correlations are utilized to provide diagnostic utilities of the present invention. In practicing the present invention, preferably the correlations sought are those between particular single

nucleotide allelic polymorphs or haplotypes thereof and the risk for developing any of the illustratively aforementioned diseases.

As referred to herein, the term "allele" or "allelic form" is intended to mean an alternative version of a gene encoding the same functional protein but containing differences in nucleotide sequence relative to another version of the same gene.

The term "allelic polymorphism" or "allelic variant" is intended to mean a variation in the nucleotide sequence within a gene, wherein different individuals in a general population express different variants of the gene.

The term "allelic pattern" is intended to mean the identity of each of the two copies of a particular gene in a patient i.e., homozygosity or heterozygosity.

The term "allelic pattern" is used herein interchangeably with "genotype."

The term "genotyping" as used herein as being the process of determining the allelic patterns of a human individual.

The term "haplotyping" as used herein as being the process of determining the linked allelic pattern within a nucleic acid strand of an individual.

PCR is used to amplify about 1 kb fragment of FasL promoter region and directly sequenced the purified PCR products. As shown in Figure 1A through 1D, four polymorphic sites within one kb of FasL promoter region are observed. The first polymorphism is at nucleotide position -844. The sequence of Genbank entries of FasL promoter at position -844 is C, but the

existence of T allele at -844 is noted in test populations. Both heterozygous (C/T) and homozygous T, as shown in Figure 1A, donors are discovered. The second polymorphism is an A to G transition at position -756, as shown in Figure 1B. The chromatogram on upper panel indicates that the donor is A homozygous at position -756, heterozygous donor with -756A/G on the middle panel, and -756G/G homozygous donor on the lower panel as shown in Figure 1B. A third polymorphic site is an A to T transversion at position -478 as shown in Figure 1C. The chromatogram on upper panel indicates that the donor is A homozygous at position -478, heterozygous donor with -478A/T on the middle panel, and -478T homozygous donor on the lower panel as shown in Figure 1C. A fourth polymorphism is a C to G transversion at position -205 as shown in Figure 1D. The chromatogram on upper panel indicates that the donor is C homozygous at position -205, heterozygous donor with -205C/G on the middle panel, and -205G homozygous donor on the lower panel as shown in Figure 1D.

Groups of SLE patients, rheumatoid arthritis (RA) patients and normal controls are genotyped for single nucleotide polymorphisms within the promoter region of Fas ligand gene. Genotype results for these three groups are summarized in Tables 1-4 for nucleotide sites -844, -756, -478 and -205, respectively. Distribution of single nucleotide polymorphs varies between the three groups. In particular, SLE patients show a statistically significant variation in genotype relative to the normal control group. Putative single nucleotide polymorph haplotypes derived from the data summarized in Tables 1-4 are shown in Table 5. A statistically significant occurrence of TAAG and

TGAG haplotypes are noted in SLE patients for nucleotides -844, -756, -478 and -205 of the Fas ligand promoter.

According to the present invention an enhancer element, C/EBP $\beta$ , in the FasL promoter region, affects the promoter activity both in the basal expression and under stimulation with PMA and ionomycin or through TCR engagement and thus has utility as a diagnostic indicative of autoimmune disease and cancer predisposition. C/EBP $\beta$  was originally identified as a mediator of IL-6 signaling, binding to IL-6-responsive elements in the promoters of acute phase response genes TNF, IL-8, and G-CSF. A. Akira et al., *EMBO Journal* 1990, 9(6):1897-906; V. Poli et al., *Cell* 1990, 63(3):643-53. Signal transduction of the acute phase response by IL-1 and LPS also induces C/EBP $\beta$  transcription. S. Akira et al., *EMBO Journal* 1990, 9(6):1897-906; T. Alam, *J. Biol. Chem.* 1992, 267(8):5021-4. The C/EBP $\beta$  element in the FasL promoter and its polymorphism potentially affect the induction of the expression of FasL in T cells. In turn, the expression difference affects the clonal deletion of auto-reactive T and B cells, and therefore, the polymorphism of C/EBP $\beta$  of FasL promoter is operative in the pathogenesis of autoimmune diseases.

The present invention utilizes the C/EBP $\beta$  role in the regulation of the FasL promoter activity as a diagnostic for SLE and other spontaneous autoimmune diseases. Because the Fas/FasL system plays a crucial role in the suppression of excessive immune responses and the maintenance of peripheral tolerance, it is reasonably assumed that the polymorphism at -844 plays a role in the pathogenesis of autoimmune diseases and is a susceptibility gene to these

diseases. The frequencies of four polymorphisms are significantly different between African American and North American Caucasians. There are significantly higher frequencies of alleles with low promoter activity in African American than in Caucasians. Since several autoimmune diseases, including

5 sarcoidosis, W.J. Arnold, In: *Kelley WN, Harris ED Jr., Ruddy S, Sledge CB(eds) Textbook of rheumatology, 4<sup>th</sup> ed.* 1993, pp.1429-1434; systemic lupus erythematosus, (SLE) P.H. Schur, In: *Kelley WN, Harris ED Jr., Ruddy S, Sledge CB (eds) Textbook of rheumatology, 4<sup>th</sup> ed.* 1993, pp. 1017-1042; and Graves' disease, T. Yanagawa et al., *Thyroid* 1996, 6(1):37-9, occur more

10 frequently in blacks than in whites. These polymorphisms are implicated in autoimmune diseases, and detection thereof according to the present invention promotes earlier therapeutic intervention and monitoring in predisposed individuals and ethnicities.

A TBE serves as an enhancer of FasL promoter in colon cancer cells

15 and is active in the FasL expression in colon cancer cells with APC mutations. The c-MYC oncogene promoter including two TBEs is identified as a target gene in this signal pathway. Expression of c-MYC was shown to be mediated through TCF/LEF1 binding sites in the c-MYC promoter, T.C. He et al., *Science* 1998, 281(5382):1509-12. There are two TCF/LEF1 binding sites in

20 FasL promoter and TCF/LEF1 binding element (TBE2) which serve as an enhancer in colon cancer cells. The present invention utilizes the sites in providing a diagnostic prior to cancerous cell proliferation. These sites are important in the expression regulation of FasL promoter activity in cancer cells.



The FasL polymorphism at position -844 is within C/EBP $\beta$  binding motif. Specifically, the C/EBP $\beta$  is active in the regulation of FasL promoter activity in the colon cancer cell lines. Natural polymorphism of C/EBP $\beta$  binding site (-844C/T) in humans affects the FasL promoter capacity.

Although nucleotide substitution at position -756A/G also significantly affects FasL promoter capacity in cancer cells, haplotype analysis indicated that the polymorphism at position -756 is linked with polymorphs at positions -844 and -205. This specific haplotype (designated as mut3 with positions of -844T, -756G, and 205G) also significantly reduces FasL promoter activity in the colon cancer cells ( $P < 0.05$ ).

The region encompassing the nucleotide position -844 is examined. TESS and MatInspector (Transcription Element Search Softwares) on the World Wide Web are used to search the candidate transcription factors that might bind the regions including nucleotide position -844. One family of proteins that has a good match is the CCAAT/enhancer binding protein family. Further search revealed that this region ATTGCGAAAT, where the underlined C is polymorphic, is in perfect match with binding site of CCAAT/enhancer binding protein beta (C/EBP $\beta$ , or nuclear factor NF-IL6) which has the consensus recognition site as 5'-T(T/G)NNGNAA(T/G)-3'. The polymorphic nucleotide is sitting in the core of C/EBP $\beta$  binding motif as shown in Figure 2A.

EMSA analysis of this 18-bp sequence confirmed that the putative C/EBP $\beta$  motive (from position -848 to -839) present in the FasL enhancer region including nucleotide position -844 indeed is C/EBP $\beta$  binding site. Lane

1 in Figure 2B shows that the consensus CCAAT enhancer binding oligos can inhibit the binding of cell nuclear extract to the labeled putative C/EBP $\beta$  motif containing oligos. Lane 2 indicates that labeled probe containing putative C/EBP $\beta$  motif binds nuclear extract. Lane 3 shows that the unlabeled probe also inhibits the binding of the nuclear extract to the labeled probe. Lane 4 shows that the anti C/EBP $\beta$  antibody supershifts the protein and probe complexes, further confirming that C/EBP $\beta$  is indeed the transcription factor that binds the region containing position -844. Lane 5 indicates that cold non-specific oligos could not inhibit the binding of labeled specific oligos. Lane 6 suggests that the labeled probe of rare allele at position -844T has much lower affinity for the transcription factor C/EBP $\beta$  than that of the common allele -844C. Taken together, the putative C/EBP $\beta$  region including -844 is indeed a C/EBP $\beta$  element, different genotypes of FasL promoter have the dramatic different affinities for the C/EBP $\beta$ .

As shown in Figure 2C, two different genotypes of FasL reporter constructs have significantly different promoter activity in SW480 cells. The promoter activity of -844C is almost twice as that of -844T construct (Luciferase light units  $6839 \pm 774$  for -844C compared  $3572 \pm 179$  for -844T). Therefore, the polymorphism at C/EBP $\beta$  binding site of FasL promoter enhancer region alters the promoter activity in colon cancer cells in vitro.

C/EBP $\beta$  (NF-IL6) transcription factor and that different alleles of the C/EBP $\beta$  element have different affinities for the transcription factor. The two different C/EBP $\beta$  binding alleles have significantly different promoter

activities in a luciferase reporter assay, with one genotype (-844C) having twice the level of activity of the other (-844T). Analysis of the distribution of the two alleles in test populations of the present invention showed an overrepresentation of the low affinity allele (-844T) of the C/EBP $\beta$  element in patients with SLE.

EMSA analysis with double-strand 18-bp oligos of Figure 4A indicate that the nuclear extracts from SW480 and Jurkat cell lines binds the radioactive labeled probe containing position -756 of FasL promoter region. Lane 1 in Figure 4B shows that labeled probe binds SW480 nuclear extract. Lane 2 shows that the unlabeled wild type probe inhibits the binding of the nuclear extract to the labeled probe. Lane 3 shows that cold non-specific oligos do not inhibit labeled wild type oligos for the binding of nuclear extracts. Lane 4 suggests that the labeled probe of mutant allele at position -756G has little binding for the unknown transcription factors. Lane 5 shows that the unlabeled mutant probe inhibits the little binding of the nuclear extract to the labeled probe. Lane 6 shows that cold non-specific oligos do not inhibit labeled mutant type oligos for the binding of nuclear extracts. Taken together, different genotypes of FasL promoter at position -756 have the dramatic different affinities for the unknown transcription factor.

As shown in Figure 4C, two different genotypes of FasL reporter constructs that different at position -756 have significantly different promoter activity in SW480 cells. The promoter activity of -756A is almost twice as that of -756G construct. Therefore, the reporter assay confirmed that the

polymorphism at nucleotide position -756 of FasL promoter enhancer region alters the promoter activity in colon cancer cells in vitro ( $P < 0.01$ ).

EMSA analysis of this 18-bp sequence of Figure 5A confirmed that the putative TCF/LEF1 motif from position -206 to -200 present in the FasL basic promoter region including nucleotide position -205 indeed is TCF/LEF1 binding site. Lane 1 in Figure 5B shows that labeled probe containing putative TCF/LEF1 motif binds SW480 nuclear extract. Lane 2 shows that the unlabeled probe inhibits the binding of the nuclear extract to the labeled probe. Lane 3 shows that the anti  $\beta$ -catenin mouse mAb supershifts and disrupts the protein and probe complexes, confirming that TCF/LEF1 and  $\beta$ -catenin complex binds the labeled oligos and the putative TCF/LEF1 is indeed the transcription factor that binds the region containing position -205. Lane 4 indicates that cold non-specific oligos do not inhibit labeled wild type oligos for the specific binding of TCF/LEF1 and  $\beta$ -catenin complexes. Lane 5 suggests that the labeled probe of mutant allele at position -205G has no binding for the transcription factors TCF/LEF1 and its complexes with  $\beta$ -catenin. Taken together, the putative TCF/LEF1 binding region including -205 is indeed a TCF/LEF1 element, different genotypes of FasL promoter have the dramatic different affinities for the TCF/LEF1.

As shown on Figure 5C, another putative TCF/LEF1 binding element (TBE1) is found between nucleotide position -838 and -832. The EMSA analyses indicate that the putative TBE is indeed TCF/LEF1 binding site. Lane 1 in Figure 5D shows that labeled probe containing putative TCF/LEF1 motif binds SW480 nuclear extract. Lane 2 shows that the unlabeled probe inhibits

the binding of the nuclear extract to the labeled probe. Lane 3 shows that the anti  $\beta$ -catenin mouse mAb supershifts and disrupts the protein and probe complexes, confirming that TCF/LEF1 and  $\beta$ -catenin complex binds the labeled oligos and the putative TCF/LEF1 is indeed the transcription factor that binds the region between nucleotide position -838 and -832. Lane 4 indicates that cold non-specific oligos do not inhibit labeled wild type oligos for the specific binding of TCF/LEF1 and  $\beta$ -catenin complexes. Lane 5 suggests that the labeled probe of mutant allele has no binding for the transcription factors TCF/LEF1 and its complexes with  $\beta$ -catenin. Taken together, the putative TCF/LEF1 binding region between nucleotide position -838 and -832 is indeed another TCF/LEF1 binding element, designated herein as TBE1.

The high mobility group (HMG) domain of TCF/LEF1 binds in a sequence specific fashion to the regulatory sequences of specific target genes;  $\beta$ -catenin supplies a transactivation domain. Thus, transcriptional activation of target genes occurs only when human TCF-4 (hTCF-4) associated with  $\beta$ -catenin. Nuclei of APC<sup>-/-</sup> colon cancer cells were found to contain a stable  $\beta$ -catenin-hTCF-4 complex that was constitutive active. Therefore, three copies of either wild type TBE2 or mutant TBE2 in pGL3-Promoter vector are constructed to test whether TBE2 site could serve as enhancer element in colon cancer with constitutive  $\beta$ -catenin-hTCF-4 complex of Figure 6A. In order to determine whether of FasL promoter could be activated in colon cancer cells and FasL gene be a target gene of APC<sup>-/-</sup>  $\beta$ -catenin-TCF signaling with the TBEs sites serving as enhancers in colon cancer cells, different reporter

constructs are engineered with mutations at TBE1 only, or TBE2 only, or mutations on both TBE sites into pGL3-Basic vector of Figure 6A. As shown in Figure 6B, TBE2 indeed could serve as an enhancer in the colon cancer cells. Three copies of wild type TBE2 could increase four times and twelve times of promoter activity over the pGL3-Promoter vector in SW620 and in SW480 respectively in luciferase assays, yet three copies of mutant TBE2 could increase as little as 1.3 times and 2.9 times of promoter activity over the pGL3-Promoter vector in SW620 and in SW480 respectively in luciferase assays. This suggests that TBE2 serves as an enhancer element in colon cancer cells and polymorphism of TBE2 (-205C/G) affects capacity of the enhancer. Although, as shown in Figure 6C, mutation of either TBE1 or TBE2 did not significantly affect the FasL promoter activity in colon cancer cells. However, destruction of both TBES significantly reduced FasL promoter activity in cancer cells (Luciferase light units  $1815 \pm 99$  for TBE1W2W (wild type),  $1816 \pm 193$  for TBE1W2M,  $1918 \pm 151$  for TBE1M2W,  $1307 \pm 77$  for TBE1M2M, and  $773 \pm 40$  for pGL3-Basic vector).

A one kb region of FasL promoter is sequenced from 254 donors. As shown on Table 7A through 7D, the distributions and gene frequencies of all polymorphic sites are significantly different between African American and North American Caucasians. Table 7A shows the distribution of C/EBP $\beta$  binding site at position -844 polymorphism in African Americans and North American Caucasians. There were significant differences in the distribution of the three genotypes, 2 X 3 contingency table,  $\chi^2 = 72.53$ ,  $P < 0.000001$ , and in allelic frequency, 2 X 2 contingency table,  $\chi^2 = 36.09$ ,  $P < 0.000001$ , between

African Americans and North American Caucasians. In Table 7B, significant differences at position -478 in the distribution of the three genotypes, 2 X 3 contingency table,  $\chi^2 = 24.88$ ,  $P < 0.00001$ , and in allelic frequency, 2 X 2 contingency table,  $\chi^2 = 7.21$ ,  $P < 0.01$ , between African Americans and North American Caucasians also have been observed. Table 7C shows significant differences at position -756 in the distribution of the three genotypes, 2 X 3 contingency table,  $\chi^2 = 24.46$ ,  $P < 0.00001$ , and in allelic frequency, 2 X 2 contingency table,  $\chi^2 = 11.40$ ,  $P < 0.001$ , between African Americans and North American Caucasians. Table 7D shows there were significant differences at position -205 in the distribution of the three genotypes, 2 X 3 contingency table,  $\chi^2 = 25.34$ ,  $P < 0.00001$ , and in allelic frequency, 2 X 2 contingency table,  $\chi^2 = 11.40$ ,  $P < 0.001$ , between African Americans and North American Caucasians.

Based on our sequence data from 254 donors, it is established that the two most common haplotypes are -844C with gene frequencies of about 64% in North American Caucasians and 20% in African American designated as wild type: -844C, -756A, and -205C and -844T with gene frequencies of about 61% in African Americans and 21% in North American Caucasians designated as -844T: -844T, -756A, and -205C. These two dominant haplotypes are different only at position -844 (C/EBP $\beta$  binding site). In addition, deduced from homozygous donors at -844, -756, and -205, it is noted that polymorphisms at above sites are linked in all homozygous donors. Therefore, the gene frequencies of mut3 haplotype -844T, -756G, and -205G of Figures 7A and 7B is the around 17% in African Americans and 2% in North American

Caucasians. PCR products are cloned from six heterozygous donors with respective heterozygous positions at -844, -756, and -205 and sequenced at least 10 clones from each donor and established existence of this specific haplotype with -844T, -756G, and -205G as the third dominant haplotype.

Furthermore, sequence data also indicate that the existence of linkage of -844T and -478 T in the FasL promoter region since majority of donors containing -478T allele are also -844T homozygous designated as DM844T478T, the fourth haplotype, but -478T is not linked with either -756G or -205G (data not shown). There are rare exceptions that -844T might link with either -756G or 205G alone (designated DM844T756G and DM844T205G, respectively). In a preliminary experiment, the constructs that are different only at position -478 are used and no difference in promoter reporter activity between -748A and -478T genotypes is noted (data not shown).

Figure 7C shows the FasL promoter activity of different haplotype constructs in colon cancer cell. Simultaneous changes of three sites with -844T, -756G, and 205G (mut3) significantly reduced FasL promoter activity in the colon cancer cells ( $P < 0.05$ ). However, double changes with either -844T and -756G (DM844T756G) or -844T and -205G (DM844T205G) lower the FasL promoter activity but the differences are not significant compared to the wild type construct ( $P = 0.06476$  for DM844T756G and  $P = 0.1937$  for DM844T205G, respectively).

Electrophoretic mobility shift assays (EMSA) of oligonucleotides incorporating the polymorphic site -205 are shown in lanes C and C' of Figure 8. The oligonucleotides primers incorporating the single nucleotide



polymorphic sites -844, -756 and -205 utilized in the EMSA of Figure 8 are shown in Table 8. EMSA is conducted as described in M. Miyamoto et al., *Cell* 1988, 54:903-913. The common -205C allele shows band retardation indicative of transcription factor binding. In contrast, the -205G allele shows no similar retardation indicating that a single nucleotide polymorph at the -205 nucleotide site markedly affects the ability of a transcription factor to bind and therefore function. The -844, -756, -478 and -205 single nucleotide polymorphs are individually operative in the regulation of apoptosis through expression of Fas ligand. The anti-inflammatory effects of the Fas ligand induced apoptosis are known. Gao et al., *J. Exp. Med.* 1998, 188:887.

The SNP at nt -844 is within the C/EBP $\beta$  (NF-IL6) transcription factor and that different alleles of the C/EBP $\beta$  element have different affinities for the transcription factor. The two different C/EBP $\beta$  binding alleles have different promoter activities in a luciferase reporter assay, with one genotype (-844C) having twice the level of activity of the other (-844T). Analysis of the distribution of the two alleles in populations of the present invention showed an overrepresentation of the low affinity allele (-844T) of the C/EBP $\beta$  element in patients with SLE.

CAAT/enhancer-binding proteins (C/EBPs) belong to a family of leucine zipper transcription factors involved in the regulation of various aspects of cellular differentiation and function in multiple tissues. Six different members of the family have been isolated and characterized (C/EBP $\alpha$  to  $\zeta$ ), all sharing a high homology in the carboxyl-terminal domain, which carries a basic DNA-binding domain and a leucine zipper motif. V. Poli, *J. Biol. Chem.*

1998, 273:29279-28282. The prototypic C/EBP, like many other transcription factors, is a modular protein, consisting of an activation domain, a DNA-binding basic region, and a leucine-rich dimerization domain. The dimerization domain, termed the "leucine zipper", is a heptad of leucine repeats that intercalate with repeats of the dimer partner, forming a coiled coil of  $\alpha$ -helices in parallel orientation. P. Agre et al., *Science* 1989, 246(4932):922-6; C.R. Vinson et al., *Genes & Development* 1993, 7(6):1047-58; W.H. Landschulz et al., *Science* 1989, 243(4899):1681-8. Electrostatic interactions between amino acids along the dimerization interface determine the specificity of dimer formation among C/EBP family members as well as with transcription factors of the NF- $\kappa$ B and Fos/Jun families. C.R. Vinson et al., *Genes & Development* 1993, 7(6):1047-58. C/EBP dimerization is a prerequisite to DNA binding. W.H. Landschulz et al., *Science* 1989, 243(4899):1681-8. DNA binding specificity, however, is determined by the DNA contact surface, the "basic" region of approximately 20 amino acids, upstream of the leucine zipper, specifically by the three amino acids lying along the protein-DNA interface (Z. Cao et al., *Genes & Development* 1991, 5(9):1538-52). Domains responsible for transcriptional activation and/or repression are located in the N-terminal end of the protein.

PCR is performed using primers to amplify a region from nucleotide -914 to +31 of CD95. The amplified product is isolated from an Agarose gel using QIAQuick Gel Extraction Kit (QIAGEN, Valencia, CA). Big Dye Terminator sequencing on an ABI 377 (PE Biosystems, Foster City, CA) is performed in the forward direction using the PCR primer at -914 (Figure 8). In

order to examine the most proximal portion of the promoter, reverse sequencing is performed using the +30 primer. Candidate SNPs are identified in the trace data as two nearly uniform florescence peaks generated by two different nucleotides as shown in Figure 8. Unlike other dye terminator chemistries, Big Dye Terminator has more uniform incorporation of labeled nucleotides that allows for the identification of heterozygous polymorphic sites. A novel SNP is identified at -690 of the proximal Fas promoter. The novel SNP at -690 is a low frequency T-C transition with a C allele frequency of <20% as shown in Table 9. In addition to this novel SNP, a low frequency G-A single nucleotide substitution is detected at nucleotide -95 (1 A/A homozygous normal individual, and 3 G/A heterozygous individuals from the SLE population). The polymorphism reported by Huang and colleagues at -670 is also detected. Q.I.R. Huang et al., *Molecular Immunology* 1997, 34:577-582. Like the new -690 SNP, the -670 SNP is confirmed by reverse sequencing using the intermediate primer as shown in Figure 10. As shown in Figure 11, this polymorphism is an A-G transition with GG homozygotes consisting of 25% of the population, AA homozygotes comprising 28% and GA heterozygotes making up 47% of the population of Table 10. The aggregate allele frequency was 0.49 for G and 0.51 for A.

Fas and FasL play a critical role in acquired immunity. This is underscored by the fact that Fas deficient mice develop lymphoproliferative syndrome, generalized lymphadenopathy, and systemic autoimmunity. P.L. Cohen et al., *Annu. Rev. Immunol.* 1991, 9:243-269. However, SLE patients have demonstrated increase in Fas expression. The same 945 bp product is

sequenced from a cohort of 31 patients with SLE to screen for novel SLE associated mutations and for the SNPs at nucleotides -670 and -690.

The -670 G/A SNP had a significant association with the SLE phenotype. Whereas, the SNP at nucleotide -690 showed a lesser association with the presence of SLE, in a normal population 25% of the individuals are GG homozygotes, 28% of the individuals are AA homozygotes, and 47% of the individuals are GA heterozygotes with an overall allele frequency of 0.49 for G and 0.51 for A. The SLE population had a significantly different genotype frequency with GG homozygotes encompassing 63% of the population, GA heterozygotes accounting for 27% and only 10% of the population being AA homozygotes ( $p < 0.006$ , as shown in Table 10). The allele frequency for SLE patients ( $G = 0.73$ ,  $A = 0.27$ ) was also different from normals  $p < 0.007$  as shown in Table 10). Even in relatively small and ethnically mixed populations an association exists between SLE and the SNP at -670.

Phenotypic differences among individuals are attributed to non-conservative nucleotide differences in coding region or to nucleotide differences in the promoter that alters gene expression. For example, a polymorphism has been characterized in the coding region of FcγRIIIa which alters the function of the receptor and associates with autoimmune disease. J. Wu et al., *J. Clin. Invest.* 1997, 100:1059-1070. Likewise, a polymorphism in FcγRIIIa segregates with African American patients with lupus nephritis. J.E. Salmon et al., *J. Clin. Invest.* 1996, 93:1348-1354. Both of these studies demonstrate the importance of single nucleotide changes on protein function.

Polymorphisms in non-coding regions of a gene also have a dramatic impact on protein function through changes in gene expression. For example, a polymorphism in the promoter of the low density lipoprotein receptor segregates with affected individuals with familial hypercholesterolemia. X.M.

- 5 Sun et al., *Human Molecular Genetics* 1995, 4:2125-2129.

Previous studies of CD95 (Fas/Apo-1) have demonstrated multiple CD95 coding region mutations in ALPS (A. Bettinardi et al., *Blood* 1997, 89:902-909; M.C. Sneller et al., *Blood* 1997, 89:1341-1348; L. Pensati et al., *Gastroenterology* 1997, 113:1384-1389; Y. Kasahara et al., *Int. Immunol.* 10 1998, 10:195-202; A.K. Vaishnav et al., *J. Clin. Invest.* 1999, 103:355-363) but not in SLE and several other autoimmune diseases (J. Wu et al., *J. Clin. Invest.* 1996, 98:1107-1113; I. Cascino et al., *Disease Markers* 1998, 13:221-225). The present invention describes a diagnostic based on an association of a SNP in the 5' promoter region of CD95 with SLE in an ethnically diverse 15 population. The location of the polymorphism is within the consensus sequence of the nuclear transcription element GAS binding site [ATTCCAG(G/A)AA]. An alteration in this site alters the regulation of CD95 expression and contributes to the altered expression seen in the SLE patients.

The correlation of various single nucleotide allelic polymorphs or 20 haplotypes thereof in individuals within the Fas or Fas ligand promoter correlate with the susceptibility of individuals to develop autoimmune diseases. It is appreciated that further single nucleotide polymorphs correlating with various infectious and autoimmune diseases exist within the Fas or Fas ligand promoter and that one skilled in the art to which the invention pertains will

readily be directed to these sites through following the teachings detailed herein. In light of the CD95 promoter and 5' promoter region sequence of the Fas ligand being known, J. Cheng et al., *J. Immunol.* 1995, 154:1239-1245 and C.J. Holtz-Heppelmann et al., *J. Biol. Chem.* 1998, 273(8):4416-4423, respectively.

In order to more fully demonstrate the advantages arising from the present invention, the following examples are set forth. It is to be understood that the following is by way of example only and is not intended as a limitation on the scope of the invention.

#### **Example 1 - Donor Protocol.**

Anti-coagulated peripheral blood is obtained from healthy normal volunteers, rheumatoid arthritis patients, and from SLE patients fulfilling the revised criteria of the American College of Rheumatology for SLE (Tan et al., \_\_\_\_\_, 1982, \_\_\_\_\_).

#### **Example 2 - Reagents.**

Anti CD3 mAb is purified from the culture supernatant from OKT3 hybridoma (ATCC, Manassas, VA). PMA is obtained from Sigma (St. Louis, MO) and ionomycin are purchased from Calbiochem (La Jolla, CA). Rabbit anti C/EBP $\beta$  polyclonal IgG is obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Transfection reagent DMRIE-C is obtained from Lifetech (Gibco, BRL). Reagents for luciferase assay are purchased from Promega (Madison, WI).

#### **Example 3 - Nucleic acid isolation.**

Genomic DNA is isolated using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Briefly, 300 µl of blood is lysed in 900 µl of the red blood lysis solution, the leukocytes are pelleted and lysed with 300 µl of Cell Lysis Solution. The RNase A solution is added to the cell lysate and incubated at 37°C for 15 min. Proteins are precipitated by adding 100 µl of Protein Precipitation solution. DNA in the supernatant is precipitated with 300 µl of 100% isopropanol and washed with 70% ethanol once.

**Example 4 - Sequencing and PCR amplification of FasL promoter region.**

The 1065 nucleotide long FasL promoter region was amplified from position -1032 to +33 with the sense primer (SEQ ID NO. 7) 5'-TTA TGC CTA TAA TCC CAG CTA CTC A-3' annealing to nucleotide position from -1032 to -1008, and anti-sense primer (SEQ ID NO. 8) 5'-CTG GGG ATA TGG GTA ATT GAA G-3' annealing to position from +12 to +33 where Figure 7A, +1 site corresponds to the A of ATG translation start codon. The PCR reaction is performed in a 9600 PCR System with 500 ng of DNA, 300 nM of each primer, 200 µM of dNTPs, 1.5 mM of MgCl<sub>2</sub>, and 2.5 U of *Taq* polymerase in a 100 µl reaction volume starting with 95°C for 5 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 45 s, extension at 72°C for 1 min with a final extension at 72°C for 7 min. All the PCR products are purified from a 2.5% agarose gel with the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The purified PCR products are sequenced from both directions with above PCR primers by using BigDye terminator sequence

kit on an ABI377 Sequencer (Applied Biosystems, Inc., Foster City, CA). The polymorphic sites are also confirmed by using M13 sequence-tagged primers for PCR amplification and dye-primer sequence strategy (Applied Biosystems, Inc., Foster City, CA). The M13-tagged sense primer (SEQ ID NO. 9) is TGT AAA ACG ACG GCC AGT CCA GCC TGG GTG ACA GAG TGA that anneals from position -933 to -913. The M13-tagged anti-sense primer (SEQ ID NO. 10) is 5'-CAG GAA ACA GCT ATG ACC TAT AGC CCT GTT AGT GTG AAC T-3' that is annealing from position -416 and -495 where underlined nucleotides are M13 forward or reverse sequences.

10 **Example 5 - FasL promoter reporter constructs.**

The FasL luciferase reporter constructs of C/EBP $\beta$  common allele (-844C) and rare allele (-844T) are generated by cloning a Kpn I/Hind III-flanked PCR products with 1026 nucleotides of FasL promoter region into pGL3-Basic vector (Promega, Madison, WI). The Kpn I/Hind III-flanked PCR products are obtained by amplifying from the genomic DNA of FasL promoter region with upper primer (SEQ ID NO. 11) 5'-GGC GGA GGT ACC CTA TAA TCC CAG CTA CTC AG-3' where underlined and bold nucleotides are Kpn I cutting site annealing at position from -1026 to -927 and lower primer (SEQ ID NO. 12) 5'-GTT CCG AAG CTT GGC AGC TGG TGA GTC AGG C-3' where underlined and bold nucleotides are Hind III cutting site annealing at position from -19 to -1. Then successive changes at nucleotide position -844, -756, -478, -205, and TBE1 (TCF/LEF binding element 1) site are made by using QuikChange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) following the vendor's instruction. For -844T construct, sense primer (SEQ ID



NO. 13) 5'-AAA TGA AAA CAT TGT GAA ATA CAA AGC AG-3' and anti-sense primer (SEQ ID NO. 14) 5'-CTG CTT TGT ATT TCA CAA TGT TTT CAT TT-3' are used. For -756G construct, sense primer (SEQ ID NO. 15) 5'-TTA ACC TGT AAG TTA TGG TGA TCG GC-3' and anti-sense primer (SEQ ID NO. 16) 5'-GCC GAT CAC CAT AAC TTA CAG GTT AA-3' are used. For -478T construct, sense primer (SEQ ID NO. 17) 5'-ATA ATG TAT AAA ATA GCA TGC AAT TA-3' and anti-sense primer (SEQ ID NO. 18) 5'-TAA TTG CAT GCT ATT TTA TAC ATT AT-3' are used. For -205G (TBE2M) construct, sense primer (SEQ ID NO. 19) 5'-AGT GAG TGG GTG TTT GTT TGA GAA GCA GAA-3' and anti-sense primer (SEQ ID NO. 20) 5'-TTC TGC TTC TCA AAC AAA CAC CCA CTC ACT-3' are used. For TBE1 mutant (TBE1M) construct, sense primer (SEQ ID NO. 21) 5'-GCG AAA TCC AAA CCA GCT-3' and anti-sense primer (SEQ ID NO. 22) 5'-AGC TGG TTT GGA TTT CGC-3' are used. All the constructs were confirmed by fluorescent automated sequencing from both directions on an ABI 377 Sequencer with ABI BigDye Terminator Cycle Sequencing Kit FS (Applied Biosystems, Inc., Foster City, CA).

**Example 6 - Generation of the triplicated FasL promoter reporter constructs.**

For the construction of TCF binding site around nucleotide position -205, four oligos were synthesized from Lifetech (Gibco, BRL). The wild type construct (-205C) is obtained by cloning into pGL3-Promoter vector (Promega, Madison, WI) the restriction enzyme *Kpn* I digested double-stranded oligos with sense strand (SEQ ID NO. 23) 5'-GGC GGA GGT ACC GTG GGT GTT

TCT TTG AGA GTG GGT GTT TCT TTG AGA GTG GGT GTT TCT TTG  
AGA **GGT ACC** TAA TGA-3' and anti-sense strand (SEQ ID NO. 24) 5'-  
TCA TTA **GGT ACC** TCT CAA AGA AAC ACC CAC TCT CAA AGA  
AAC ACC CAC TCT CAA AGA AAC ACC CAC **GGT ACC** TAA TGA-3'.

- The mutant construct (-205G) is obtained by cloning into pGL3-promoter the restriction enzyme *Kpn* I digested double-stranded oligos with sense strand (SEQ ID NO. 25) 5'-GGC GGA **GGT ACC** GTG GGT GTT TGT TTG AGA GTG GGT GTT TGT TTG AGA GTG GGT GTT TGT TTG AGA **GGT ACC** TAA TGA -3' and anti-sense strand (SEQ ID NO. 26) 5'-TCA TTA **GGT ACC** TCT CAA ACA AAC ACC CAC TCT CAA ACA AAC ACC CAC TCT CAA ACA AAC ACC CAC **GGT ACC** TAA TGA-3' where underlined and bold nucleotides are *Kpn* I cutting site.

### Example 7 - SLE correlation experimental techniques.

**Transient transfection, Cell stimulation and luciferase assay:** The

- 15 Jurkat human leukemic T cell line is maintained in the RPMI supplemented  
with 10% fetal calf serum, penicillin (1000 units/ml), streptomycin (1000  
units/ml), and glutamine (20 mM). Each transient transfection experiment is  
carried out with  $2 \times 10^6$  Jurkat cells and  $2 \mu\text{g}$  construct plasmid DNA and  $0.5$   
 $\mu\text{g}$  of pCMV.SPORT- $\beta\text{gal}$  plasmid purified with Wizard PureFection Plasmid  
20 DNA Purification System (Promega, Madison, WI) by using  $4 \mu\text{l}$  of DMRIE-C  
reagent following vendor's instruction (GIBCO BRL). Transfected cells are  
cultured in anti-CD3 coated wells in 6 well plates, each well was treated with  $1$   
 $\text{ml}$  of  $20 \mu\text{g/ml}$  anti-CD3 mAb by incubating at  $37^\circ\text{C}$  for two hours and then  
washing with  $3 \text{ ml}$  of RPMI 1640 without FBS, or stimulated with  $50 \mu\text{g/ml}$

PMA plus 1  $\mu\text{M}$  ionomycin (Calbiochem, CA), or without any stimulation for 20 hours after starting of transfection procedure. The harvested cells are washed twice with PBS (pH 7.4) and are lysed in 300  $\mu\text{l}$  of 1x report lysis buffer (Promega, Madison, WI). Cell debris is removed by centrifugation and the supernatant is used in the luciferase assay using a Monolight 2010 luminometer (Promega, Madison, WI).

**Nuclear extract preparations:** Jurkat cells ( $3.5 \times 10^6$  /ml) are stimulated with 10  $\mu\text{g/ml}$  of LPS for two hours or cultured on anti-CD3 mAb coated plates for 3.5 hours before nuclear extraction. Cells are washed with PBS (pH 7.4) and resuspended in buffer A (10 mM Hepes, 1.5 mM  $\text{MgCl}_2$ , and 10 mM KCl in the presence of protease inhibitors and dithiothreitol (100  $\mu\text{M}$ ) and lysed by the addition of Nonidet P-40 to a final concentration of 0.1%. Nuclei are pelleted and washed in buffer A, and nuclear protein is extracted in buffer C (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , and 0.2 mM EDTA). After pelleting the nuclear debris, the supernatant is removed and diluted with equal volume of Buffer D (20 mM Hepes, 20% glycerol, 50 mM KCl, and 0.2 mM EDTA). Protein concentration is determined by measuring  $\text{OD}_{280}$ .

**Electrophoretic mobility shift assay:** For each binding reaction, 8  $\mu\text{g}$  of nuclear extract is incubated in 1 X binding buffer (4% glycerol, 1 mM  $\text{MgCl}_2$ , 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50  $\mu\text{g/ml}$  poly(dI-dC)poly(dI-dC)) with  $^{32}\text{P}$  labeled oligos. The following double-stranded oligonucleotide probes are used in these experiments: C/EBP consensus, (SEQ ID NO. 27) 5'-TGC AGA TTG CGC AAT CTG CA-3';

- nonspecific, (SEQ ID NO. 27) 5'- TGC AGA TTG CGC AAT CTG CA -3'; C/EBP $\beta$  common allele (-844C), (SEQ ID NO. 28) 5'-AAA ACA TTG CGAAAT ACA-3'; C/EBP $\beta$  rare allele (-844T), (SEQ ID NO. 29) 5'-AAA ACA TTG TGAAAT ACA-3' where the polymorphic nucleotide -844 is underlined
- 5 in Figure 3. C/EBP $\beta$  supershift assay was carried out by following vendor's instruction (Santa Cruz Biotechnology, Santa Cruz, CA).

**Example 8 - Cancer correlation experimental techniques.**

- Transient transfection and luciferase assay:** The colon cancer cell lines SW480 and SW620 are obtained from ATCC (Manassas, VA) and are maintained in L-15 medium supplemented with 10% fetal calf serum and L-glutamine (2 mM) and grow in monolayer without CO<sub>2</sub>. Each transient transfection experiment is carried out on 6-well plates with 2 X 10<sup>5</sup> SW480 cancer cells in each well and 0.5  $\mu$ g construct plasmid DNA and 0.05  $\mu$ g of pCMV.SPORT- $\beta$ gal plasmid purified with Wizard PureFection Plasmid DNA Purification System (Promega, Madison, WI) by using 4  $\mu$ l of DMRIE-C reagent following vendor's instruction (GIBCO BRL). Transfected cells are cultured for 20 hours after starting of transfection procedure. The cells in the wells are washed twice with PBS (pH 7.4) and are lysed in 500  $\mu$ l of 1X report lysis buffer (Promega, Madison, WI). The cancer cells are scraped and transferred to microfuge tubes and vortex for 10 sec to break and lyse cells. Cell debris is removed by centrifugation and the supernatant is used in the luciferase assay using a Monolight 2010 luminometer (Promega, Madison, WI). Luciferase light units, standardized to beta-galactosidase activity, are reported as the mean of triplicate samples.
- 10
- 15
- 20

**Nuclear extract preparations:** SW480 cancer cell are grown to monolayer and harvested by treating with 0.25% trypsin and 0.02% EDTA. Jurkat cells ( $3.5 \times 10^6$  /ml) are stimulated with 10  $\mu$ g/ml of LPS for two hours or cultured on anti-CD3 mAb coated plates for 3.5 hours before nuclear extraction. Cells ( $10^7$ ) are washed with PBS (pH7.4) and resuspended in buffer A (10mM Hepes, 1.5 mM  $MgCl_2$ , and 10 mM KCl in the presence of protease inhibitors and dithiothreitol (100  $\mu$ M) and lyzed by the addition of Nonidet P-40 to a final concentration of 0.1%. Nuclei are pelleted and washed in buffer A, and nuclear protein is extracted in buffer C (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM  $MgCl_2$ , and 0.2mM EDTA). After pelleting the nuclear debris, the supernatant is removed and diluted with equal volume of Buffer D (20 mM Hepes, 20% glycerol, 50 mM KCl, and 0.2 mM EDTA). Protein concentration is determined by measuring  $OD_{280}$ .

**Electrophoretic mobility shift assay:** For each binding reaction, 8  $\mu$ g of nuclear extract is incubated in 1 X binding buffer (4% glycerol, 1 mM  $MgCl_2$ , 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH7.5, and 50  $\mu$ g/ml poly(dI-dC)poly(dI-dC)) with  $^{32}P$  labeled oligos in a volume of 10  $\mu$ l. Binding reactions are incubated at room temperature for 30 min with 50,000 cpm (0.1-0.5 ng) of double stranded oligonucleotides end-labeled with [ $\gamma$ - $^{32}P$ ]ATP using T4 polynucleotide kinase. Unlabeled specific or non-specific competitor oligonucleotides are used where indicated at a 200-fold excess. Protein/DNA complexes and unbound DNA probe are then resolved on 5% non-denaturing polyacrylamide gel and visualized by autoradiography. The following double-stranded oligonucleotide probes are used in these

experiments: 1) C/EBP consensus, (SEQ ID NO. 27) 5'-TGC AGA TTG CGC AAT CTG CA-3'; nonspecific, (SEQ ID NO. 28) 5'-GTG GGT GTT TGT TTG AGA-3'; C/EBPβ -844C allele, (SEQ ID NO. 1) 5'-AAA ACA TTG CGA AAT ACA-3'; C/EBPβ -844T allele, (SEQ ID NO. 29) 5'-AAA ACA TTG TGA AAT ACA-3' where polymorphic nucleotide position -844 is underlined as shown in Figure 2A. C/EBPβ supershift assay was carried out by following vendor's instruction (Santa Cruz Biotechnology, Santa Cruz, CA). 2) -756A, (SEQ ID NO. 3) 5'-ACC TGT AAA TTA TGG TGA-3'; -756G, (SEQ ID NO. 30) 5'-ACC TGT AAG TTA TGG TGA-3' where polymorphic nucleotide position -756 is underlined; Oct1 consensus, (SEQ ID NO. 31) 5-TGT CGA ATG CAA ATC ACT AGA A-3'; nonspecific, (SEQ ID NO. 27) 5'-TGC AGA TTG CGC AAT CTG CA-3' as shown in Figure 4A. 3) -205C (TBE2 wild type), (SEQ ID NO. 5) 5'-GTG GGT GTT TCT TTG AGA-3'; -205G (TBE2 mutant), (SEQ ID NO. 28) 5'-GTG GGT GTT TGT TTG AGA-3' where polymorphic nucleotide position -205 is underlined; nonspecific, (SEQ ID NO. 29) 5'-AAA ACA TTG TGA AAT ACA-3' (Figure 5A). 4) TBE1 wild type, (SEQ ID NO. 32) 5'-GCG AAA TAC AAA GCA GCT-3'; TBE1 mutant, (SEQ ID NO. 33) 5'-GCG AAA TCC AAA CCA GCT-3' where the underlined nucleotides are introduced mutation sites; nonspecific, (SEQ ID NO. 29) 5'-AAA ACA TTG TGA AAT ACA-3' as shown in Figure 5C. TCF/β-catenin complex supershift assay is carried out by following vendor's instruction (Transduction Laboratories, Lexington, KY).

**Example 9 - PCR product cloning and haplotype determination.**

In order to verify the existence of different haplotypes and genotypes of FasL promoter, gel purified PCR product is directly cloned into pGEM-T Easy Vector (Promega Cor., Madison, WI) following vendor's manual. 10 clones from each of 6 heterozygous donors are sequenced on an ABI 377 Sequencer with ABI Dye Terminator Cycle Sequencing Kit FS (Applied Biosystems, Inc., Foster City, CA).

**Example 10 - Statistical analysis.**

The  $\chi^2$  tests are used to analyze the distribution of FasL promoter genotypes and gene frequencies in African Americans and North American Caucasians. Differences in FasL promoter activities of various constructs are analyzed by Student's *t* test. The null hypothesis is rejected at the 95% confidence level ( $P < 0.05$ ).

Patent applications and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. These applications and publications are incorporated herein by reference to the same extent as if each individual application or publication was specifically and individually incorporated herein by reference.

The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. It is the following claims, including all equivalents thereof, which define the scope of the invention.

TABLES 1 and 2

Table 1 - The distribution of putative nuclear factor-1 binding site (nt -844) polymorphism within the promoter region of fas ligand gene in SLE, RA patients, and normal controls

	SLE patients n = 62	RA patients n = 40	Normal controls n = 39
Genotype			
No. of subjects (% of group)			
CC	14 (23%)	22 (55%)	18 (46%)
CT	27 (43%)	14 (35%)	14 (36%)
TT	21 (34%)	4 (10%)	7 (18%)
Allelic frequency			
C	0.44	0.73	0.64
T	0.56	0.27	0.36

Putative NF-1 binding site is ATTGg (from -848 to -844).

Table 2 - The distribution of putative TATA box (-478) polymorphism within the promoter region of fas ligand gene in SLE, RA patients, and normal controls

	SLE patients n = 62	RA patients n = 40	Normal controls n = 39
Genotype			
No. of subjects (% of group)			
AA	57 (92%)	30 (75%)	32 (82%)
AT	4 (6%)	7 (18%)	5 (13%)
TT	1 (2%)	3 (8%)	2 (5%)
Allelic frequency			
A	0.95	0.83	0.88
T	0.05	0.17	0.12

Putative TATA box is TATAAAAaA (from -485 to -477).



## TABLES 3 and 4

Table 3 - The distribution of putative IRF-1 or IRF-2 (-756) polymorphism within the promoter region of fas ligand gene in SLE, RA patients, and normal controls

	SLE patients n = 62	RA patients n = 40	Normal controls n = 39
Genotype			
No. of subjects (% of group)			
AA	54 (87%)	40 (100%)	38 (97%)
AG	5 (8%)	0 (0%)	1 (3%)
GG	3 (5%)	0 (0%)	0 (0%)
Allelic frequency			
A	0.91	1.00	0.99
G	0.09	0.00	0.01

Putative IRE-1 or IRE-2 site is AAgTTA (from -758 to -753).

Table 4 - The distribution of putative ICSBP site (-205) polymorphism within the promoter region of fas ligand gene in SLE, RA patients, and normal controls

	SLE patients n = 62	RA patients n = 40	Normal controls n = 39
Genotype			
No. of subjects (% of group)			
CC	54 (87%)	40 (100%)	38 (97%)
CG	5 (8%)	0 (0%)	1 (3%)
GG	3 (5%)	0 (0%)	0 (0%)
Allelic frequency			
C	0.91	1.00	0.99
T	0.09	0.00	0.01

The core sequence for ICSBP binding site is NNNTTTc (from -212 to -205).

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TABLE 5

Putative haplotypes for SNPs of the 5' Fas ligand promoter region

<u>n=78</u> <u>NL - Normal</u> <u>Controls</u>	<u>n=80</u> <u>RA</u> <u>Patients</u>	<u>n=122</u> <u>SLE</u> <u>Patients</u>	<u>-844</u>	<u>-756</u>	<u>-478</u>	<u>-205</u>
49 (63%)	58 (73%)	54 (44%)	C	A	A	C
19 (24%)	9 (11%)	51 (42%)	T	A	A	C
9 (12%)	13 (17%)	6 (5%)	T	A	T	C
0 (0%)	0 (0%)	1 (1%)	T	A	A	G
1 (1%)	0 (0%)	10 (8%)	T	G	A	G

09507501-050001

TABLE 8

FasI Promoter Primers for EMSA:

Pair 1 (underlined letter indicates the polymorphic site at -844):

1. 5'-AAA ACA TTG CGA AAT ACA-3' SEQ ID NO. 1
2. 5'-TGT ATT TCG CAA TGT TTT-3' SEQ ID NO. 2

Pair 2 (underlined letter indicates the polymorphic site at -756):

1. 5'-ACC TGT AAA TTA TGG TGA-3' SEQ ID NO. 3
2. 5'-TCA CCA TAA TTT ACA GGT-3' SEQ ID NO. 4

Pair 3 (underlined letter indicates the polymorphic site at -205):

1. 5'-GTG GGT GTT TCT TTG AGA-3' SEQ ID NO. 5
2. 5'-TCT CAA AGA AAC ACC CAC-3' SEQ ID NO. 6

00007531 052304  
102250 102250

TABLE 9

Distribution of CD95 alleles at nucleotide -690 in SLE patients and non-SLE controls

	Non-SLE controls (n=36)	SLE patients (n=30)
<b>Genotype</b>		
nt -690 T/T	30 (83%)	26 (87%)
nt -690 C/T	6 (17%)	4 (13%)
nt -690 C/C	0 (0%)	0 (0%)
<b>Allelic Frequency</b>		
nt -690 T	0.92	0.93
nt -690 C	0.08	0.07

Regions of the distal promoter of CD95 are PCR amplified and subjected to direct sequencing as shown in Figure 2. Allele and gene frequencies of a novel polymorphism at nt -690 in 30 ethnically diverse patients and 36 ethnically diverse normal volunteers are shown.

p>0.05, Normal controls vs. SLE patients.

TABLE 10

Distribution of CD95 alleles at nucleotide -670 in SLE patients and non-SLE controls

patients (n=30)	Non-SLE controls (n=36)	SLE
<b>Genotype*</b>		
nt -670 A/A (10%)	10 (28%)	3
nt -670 A/G (27%)	17 (47%)	8
nt -670 G/G (63%)	9 (25%)	19
<b>Allelic Frequency**</b>		
nt -670 A 0.27	0.51	
nt -670 G 0.73	0.49	

Regions of the distal promoter of CD95 are PCR amplified and subjected to direct sequencing as described in Table 7. Allele and gene frequencies of the polymorphism at nt -670 in 30 ethnically diverse patients and 36 ethnically diverse normal volunteers are shown.

\*SLE patients vs. normal controls; 3 x 2 contingency table,  $\chi^2 = 10.12$ ,  $P < 0.006$ .

\*\*SLE patients vs. normal controls; 2 x 2 contingency table,  $\chi^2 = 7.35$ ,  $P < 0.007$ .

Claims

- 1           1.     A diagnostic method for determining autoimmune disease or  
2     cancer susceptibility comprising the step of:  
3           haplotyping an individual in a Fas ligand promoter region.
- 1           2.     The method of claim 1 wherein haplotyping occurs at a  
2     polymorph in the Fas ligand promoter region.
- 1           3.     The method of claim 2 wherein said polymorph is active in  
2     binding NF-IL6 transcription factor.
- 1           4.     The method of claim 2 wherein said polymorph is active in  
2     binding TCF/LEF-1.
- 1           5.     The method of claim 2 wherein haplotyping comprises the  
2     polymorph selected from a group consisting of: -844, -756, -478 and -205.
- 1           6.     A method for identifying susceptibility to a disease, said method  
2     comprising:  
3           identifying a first Fas ligand promoter genotype at a nucleotide site of  
4     an individual;  
5           quantifying susceptibility of said individual to the disease; and  
6           comparing susceptibility of said individual to the disease to  
7     susceptibility of a second individual, said second individual having a second

8 Fas ligand promoter genotype, the second Fas ligand promoter genotype being  
9 dissimilar from the first Fas ligand promoter genotype.

1 7. The method of claim 1 wherein said nucleotide site is -844.

1 8. The method of claim 1 wherein said nucleotide site is -756.

1 9. The method of claim 1 wherein said nucleotide site is -478.

1 10. The method of claim 1 wherein said nucleotide site is -205.

1 11. The method of claim 1 wherein said nucleotide site binds NF-  
2 IL6 transcription factor.

1 12. The method of claim 1 wherein said nucleotide site binds  
2 TCF/LEF-1.

1 13. A Fas ligand promoter single nucleotide polymorph.

1 14. The Fas ligand promoter of claim 13 wherein said single  
2 nucleotide polymorph is -844 C/T.

1 15. The Fas ligand promoter of claim 13 wherein said single  
2 nucleotide polymorph is -756 A/G.

1           16. The Fas ligand promoter of claim 13 wherein said single  
2 nucleotide polymorph is -478 C/T.

1           17. The Fas ligand promoter of claim 13 wherein said single  
2 nucleotide polymorph is -205 C/G.

1           18. A diagnostic Fas ligand promoter primer comprising a  
2 nucleotide sequence selected from a group consisting of SEQ ID numbers 1, 2,  
3 3, 4, 5 and 6.

1           19. A test kit for disease susceptibility comprising: reagents for  
2 assaying for a single nucleotide polymorph within a Fas ligand promoter gene  
3 of an individual together with instructions for the use thereof as a diagnostic.

1           20. Use of a single nucleotide polymorph within a Fas ligand  
2 promoter gene of an individual for determining susceptibility of said individual  
3 to a disease.

1           21. The use of claim 19 wherein said disease is selected from a  
2 group consisting of autoimmune disease and non-lymphatic cancer.

1           22. The single nucleotide polymorphism according to claim 13 as  
2 described herein in any of the examples.



1           23.   The method according to claim 1 as described herein in any of  
2           the examples.

1           24.   A diagnostic method for determining autoimmune disease or  
2           cancer susceptibility comprising the step of:  
3           haplotyping an individual in a Fas promoter region.

1           25.   The method of claim 1 wherein haplotyping occurs at a  
2           polymorphism in the Fas promoter region.

1           26.   The method of claim 1 wherein haplotyping comprises the  
2           polymorph selected from a group consisting of: -690 and -95.

1           27.   A Fas promoter single nucleotide polymorph located at a  
2           nucleotide site greater than -660 or less than -680.

1           28.   The Fas promoter of claim 27 wherein said single nucleotide  
2           polymorphism is -690 T/C.

1           29.   The Fas promoter of claim 27 wherein said single nucleotide  
2           polymorphism is -95 G/A.

1           30. A test kit for disease susceptibility comprising: reagents for  
2           assaying for a single nucleotide polymorph within a Fas promoter gene of an  
3           individual together with instructions for the use thereof as a diagnostic.

1           31. Use of a single nucleotide polymorph within a Fas promoter  
2           gene of an individual for determining susceptibility of said individual to a  
3           disease.

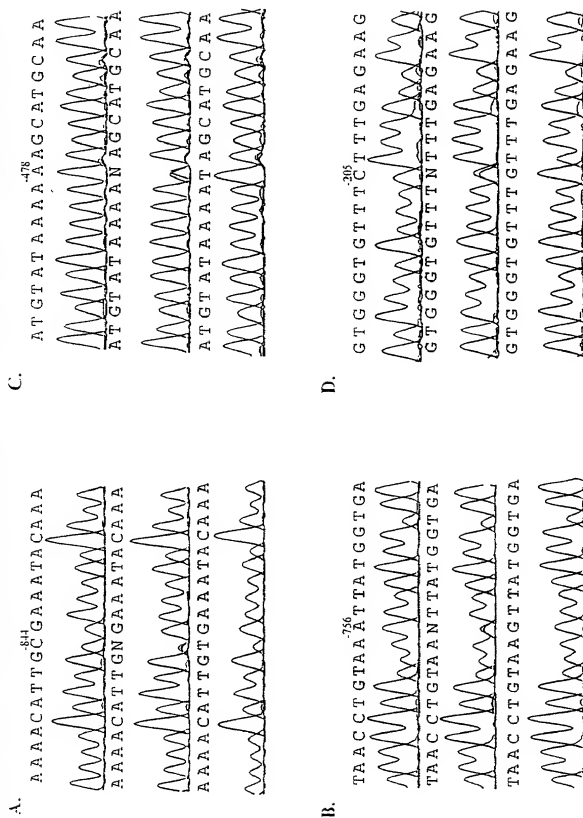
1           32. A method for identifying susceptibility to a disease, said method  
2           comprising:  
3           identifying a first Fas promoter genotype in a nucleotide site of an  
4           individual;  
5           quantifying susceptibility of said individual to the disease; and  
6           comparing susceptibility of said individual to the disease to  
7           susceptibility of a second individual, said second individual having a second  
8           Fas promoter genotype, the second Fas promoter genotype being dissimilar  
9           from the first Fas promoter genotype.

10



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/23623</b> <b>(43) International Publication Date:</b> 27 April 2000 (27.04.00)
<b>(21) International Application Number:</b> PCT/US99/24148 <b>(22) International Filing Date:</b> 15 October 1999 (15.10.99)  <b>(30) Priority Data:</b> 60/104,644 16 October 1998 (16.10.98) US 60/139,659 17 June 1999 (17.06.99) US  <b>(71) Applicant (for all designated States except US):</b> UAB RESEARCH FOUNDATION [US/US]; The University of Alabama at Birmingham, 1120-G Administration Building, 701 South 20th Street, Birmingham, AL 35294-0111 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> <u>KIMBERLY</u> , Robert, P. [-US]; 1900 University Boulevard, 429 THT, Birmingham, AL 35204-0006 (US).  <b>(74) Agents:</b> COGEN, Ellen, S. et al.; Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C., Suite 400, 280 N. Old Woodward Avenue, Birmingham, MI 48009-5394 (US).		<b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> POLYMORPHISM IN Fas PROMOTER AND Fas LIGAND PROMOTER		
<b>(57) Abstract</b>  The use of a single nucleotide polymorph in a Fas ligand or Fas promoter region to diagnose disease is disclosed. The correlation of single nucleotide polymorphs in either a Fas ligand or Fas promoter region is correlated to individual susceptibility to autoimmune diseases or non-lymphatic cancer.		



FIGURES 1A-1D

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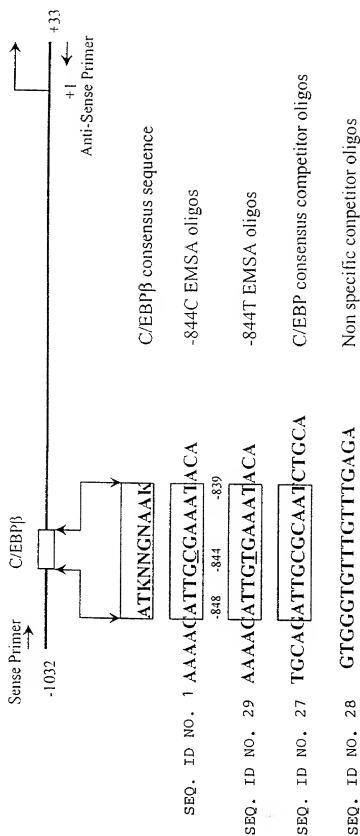


FIGURE 2A

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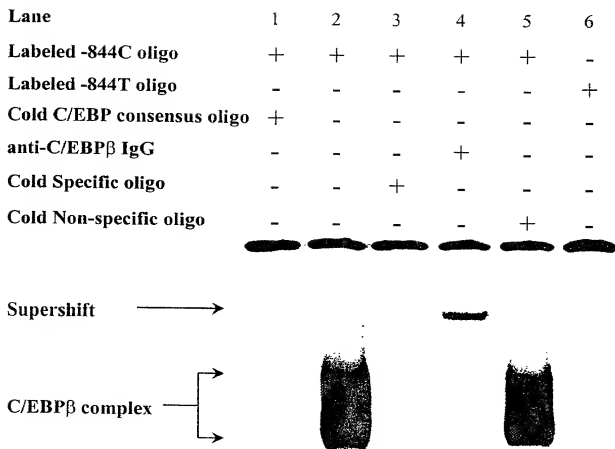


FIGURE 2B

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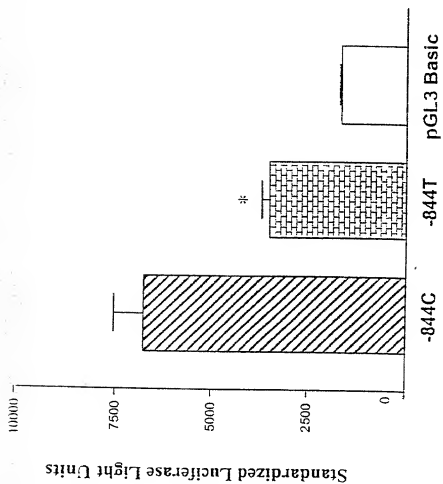


FIGURE 2C

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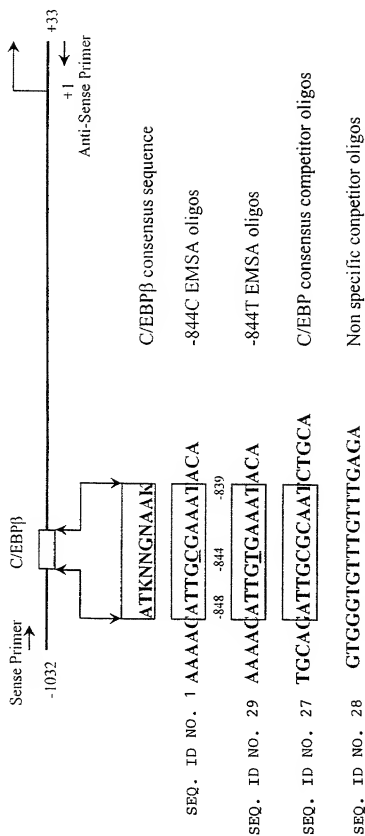


FIGURE 3



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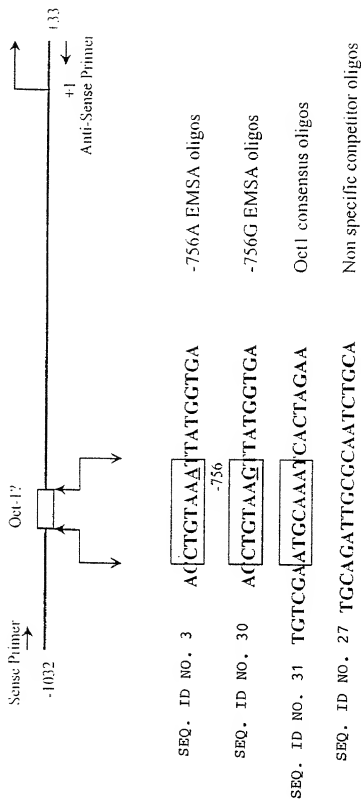


FIGURE 4A

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Lane	1	2	3	4	5
Labeled -756A oligo	+	+	+	+	-
Labeled -756G oligo	-	-	-	-	+
Cold Oct-1 consensus oligo	+	-	-	-	-
Cold Specific oligo	-	-	+	-	-
Cold Non-specific oligo	-	-	-	+	-

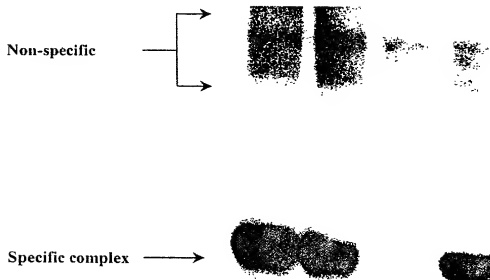


FIGURE 4B

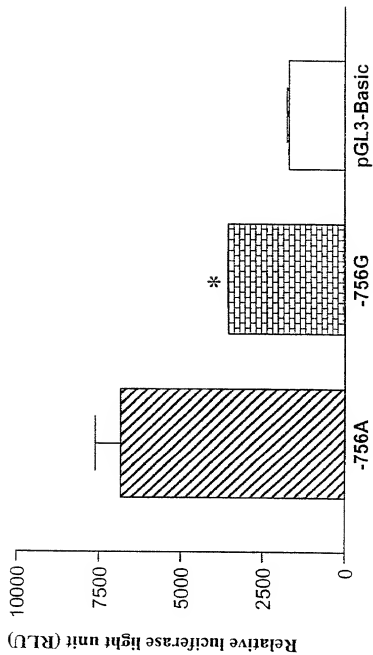


FIGURE 4C

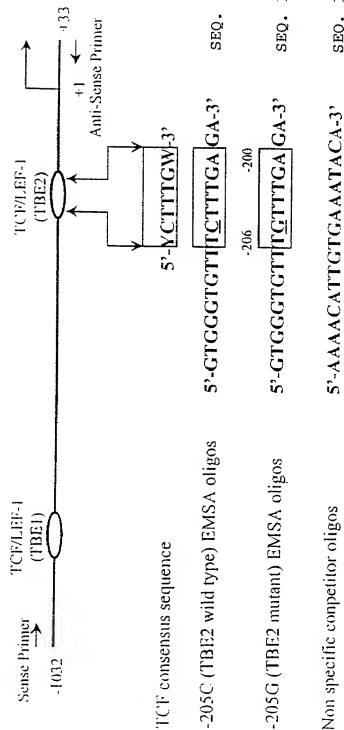


FIGURE 5A

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Lane	1	2	3	4	5
Labeled -205C oligo	+	+	+	+	-
Labeled -205G oligo	-	-	-	-	+
Anti- $\beta$ -catenin mAb	-	-	+	-	-
Cold Specific oligo	-	+	-	-	-
Cold Non-specific oligo	-	-	-	+	-

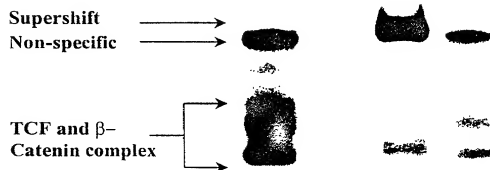


FIGURE 5B

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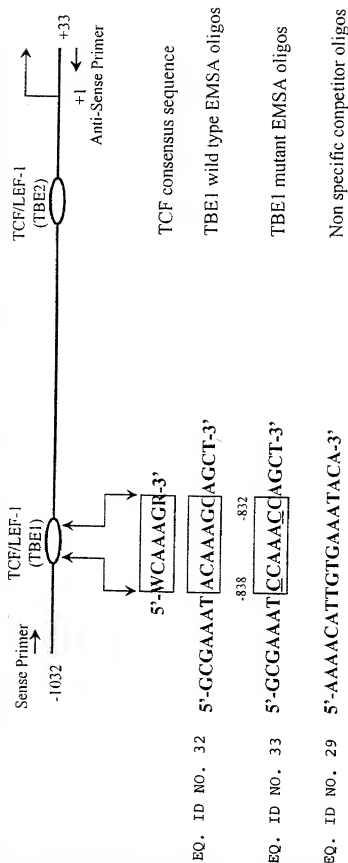


FIGURE 5C

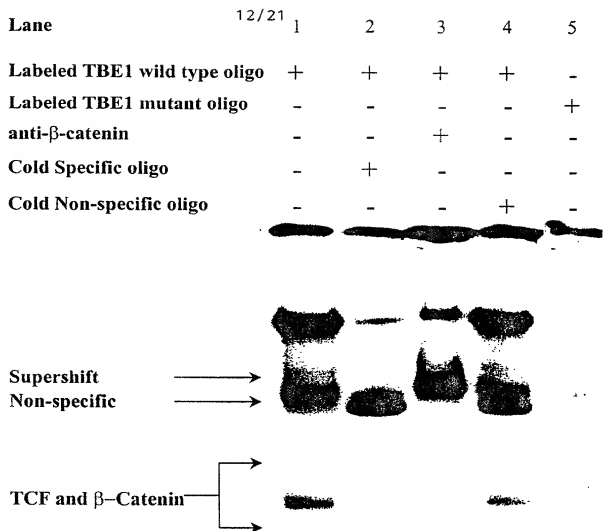


FIGURE 5D

T0540860-T0540860

PCT/US99/24148

WO 00/23623

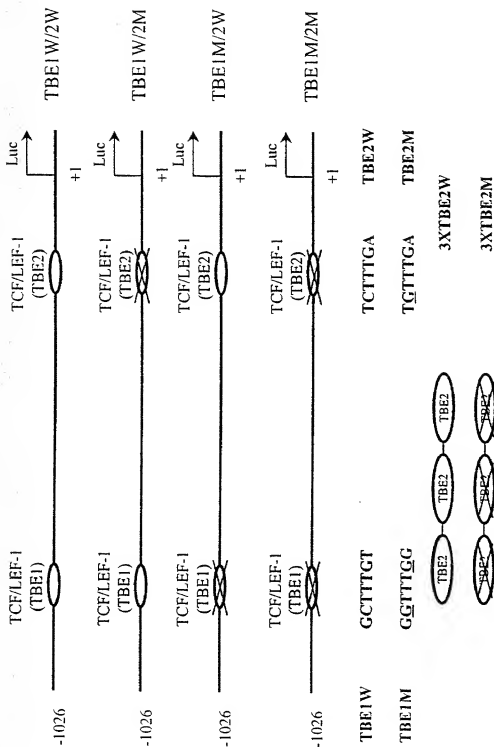


FIGURE 6A



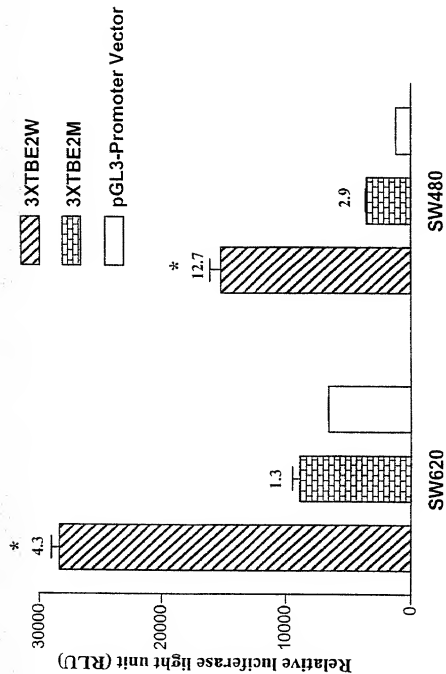


FIGURE 6B

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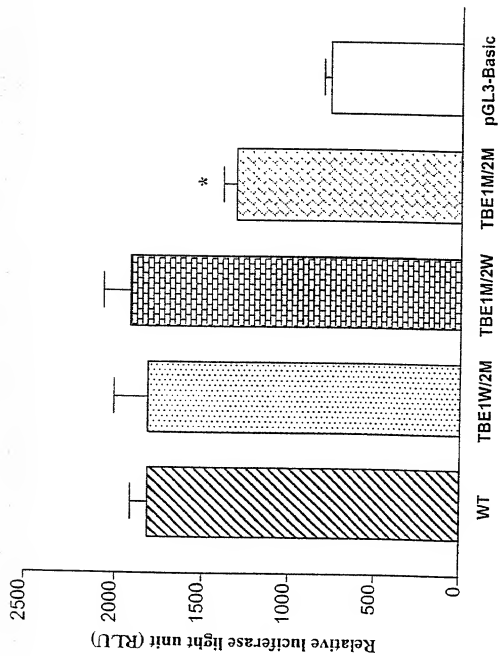
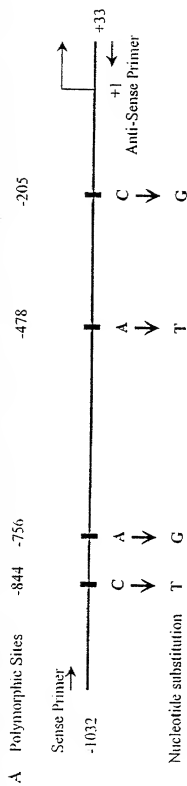


FIGURE 6C



B

	-844	-756	-478	-205	Designation
	C	A	A	C	WT (-844C)
	T	A	A	C	-844T
	T	G	A	G	MUT3
	T	A	T	C	DM844T478T
	T	G	A	C	DM844T756G
	T	A	A	G	DM844T205G

FIGURE 7A - 7B

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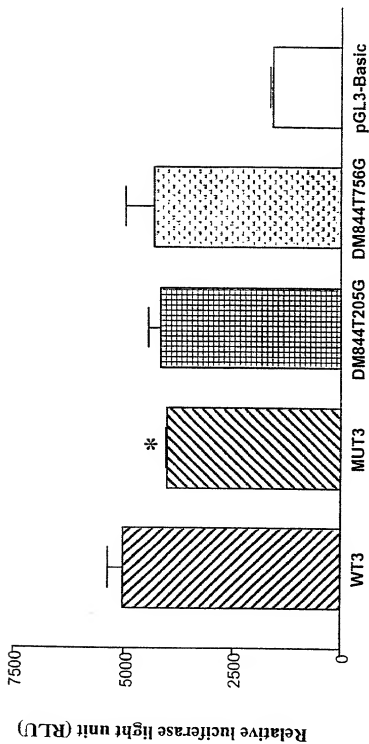
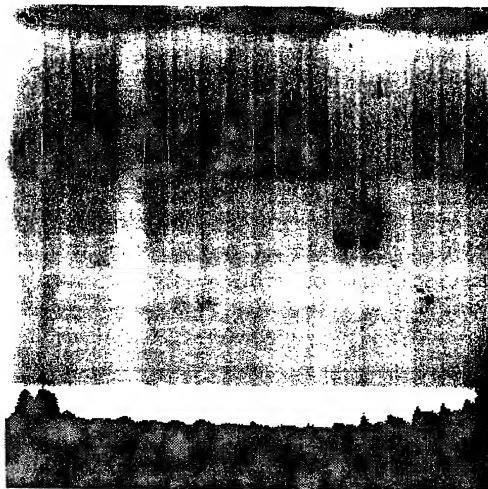


FIGURE 7C

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Cell Type: HT29

	-844C	-844T	-756A	-756G	-205C	-205G
	<u>A</u>	<u>A'</u>	<u>B</u>	<u>B'</u>	<u>C</u>	<u>C'</u>
Competitor	-	-	s	-	-	-
IFN- $\gamma$	+	+	+	+	+	+
	-	-	ns	-	-	-
	+	+	+	+	+	+
	-	-	s	-	-	-
	+	+	+	+	+	+
	-	-	ns	-	-	-
	+	+	+	+	+	+
	-	-	s	-	-	-
	+	+	+	+	+	+



S indicates homologous specific oligos

NS stands for an unrelated non-specific probe (NFI6)

FIGURE 8

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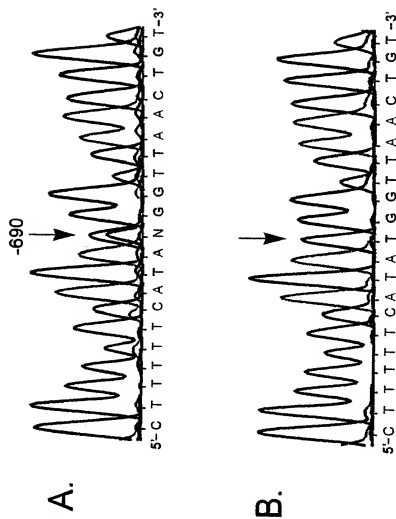
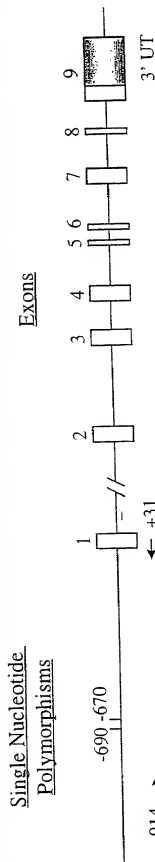


FIGURE 9



PCR Amplification/Sequencing Primers

-914: 5'	ACA GTG TTC ACC AGA GCA CGA A	3''
-594: 5'	CCC CGC AAG TCT TTC TCT GAG T	3'
-340: 5'	GGG TAG GGA GGG GGA GAA G	3'
+31 5'	CCA GAG GTA GGA GGG TCC AGA T	3'

FIGURE 10

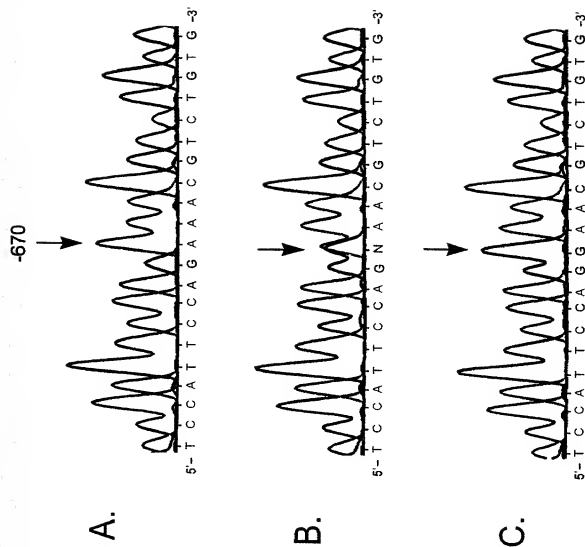


FIGURE 11



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: The UAB Research Foundation
- (ii) TITLE OF INVENTION: POLYMORPHISM IN Fas PROMOTER and Fas LIGAND PROMOTERS
- (iii) NUMBER OF SEQUENCES: 33
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Ellen S. Cogen  
Gifford, Krass, Groh, Sprinkle,  
Anderson & Citkowski, P.C.
- (B) STREET: 280 N. Old Woodward Ave., Suite 400
- (C) CITY: Birmingham
- (D) STATE: Michigan
- (E) COUNTRY: U.S.
- (F) ZIP: 48009
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM
- (C) OPERATING SYSTEM: Windows 98
- (D) SOFTWARE: Microsoft Word 2000
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Ellen S. Cogen
- (B) REGISTRATION NUMBER: 38,109
- (C) REFERENCE/DOCKET NUMBER: UAB-14452/22
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 248-647-6000
- (B) TELEFAX: 248-647-5210

## (2) INFORMATION FOR SEQ ID NO. 1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18
- (B) TYPE: Nucleic acid

- (C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

AAA ACA TTG CGA AAT ACA 18

(3) INFORMATION FOR SEQ ID NO. 2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

TGT ATT TCG CAA TGT TTT 18

(4) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:

(A) Description: Other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:

(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

ACC TGT AAA TTA TGG TGA 18

(5) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:

(A) Description: Other nucleic acid

(iii) HYPOTHETICAL: No

09507501.000001

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:

- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

TCA CCA TAA TTT ACA GGT 18

(6) INFORMATION FOR SEQ ID NO. 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: Nucleic acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:

- (A) Description: Other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:

- (B) STRAIN:
- (C) INDIVIDUAL ISOLATE:
- (D) DEVELOPMENTAL STAGE:
- (F) TISSUE TYPE:
- (G) CELL TYPE:
- (H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 5:

GTG GGT GTT TCT TTG AGA 18

(7) INFORMATION FOR SEQ ID NO. 6:

00007501-052304  
10/22/99 10:54:08 AM

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(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 6:  
TCT CAA AGA AAC ACC CAC 18

(8) INFORMATION FOR SEQ ID NO. 7:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:

- (G) CELL TYPE:  
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 7:

TTA TGC CTA TAA TCC CAG CTA CTC A

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(9) INFORMATION FOR SEQ ID NO. 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:

- (A) Description: Other nucleic acid

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: Yes

(vi) ORIGINAL SOURCE:

- (B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 8:

CTG GGG ATA TGG GTA ATT GAA G

22

(10) INFORMATION FOR SEQ ID NO. 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE:

- (A) Description: Other nucleic acid

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- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 9:

TGT AAA ACG ACG GCC AGT CCA GCC TGG GTG  
ACA GAG TGA

39

(11) INFORMATION FOR SEQ ID NO. 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40
  - (B) TYPE: Nucleic acid
  - (C) STRANDEDNESS: Double
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:
  - (A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: Yes
- (vi) ORIGINAL SOURCE:
  - (B) STRAIN:
  - (C) INDIVIDUAL ISOLATE:
  - (D) DEVELOPMENTAL STAGE:
  - (F) TISSUE TYPE:
  - (G) CELL TYPE:
  - (H) CELL LINE:

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 10:

CAG GAA ACA GCT ATG ACC TAT AGC CCT GTT  
AGT GTG AAC T

40

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## (12) INFORMATION FOR SEQ ID NO. 11:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:  
(C) INDIVIDUAL ISOLATE:  
(D) DEVELOPMENTAL STAGE:  
(F) TISSUE TYPE:  
(G) CELL TYPE:  
(H) CELL LINE:

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO. 11:

GGC GGA GGT ACC CTA TAA TCC CAG CTA CTC AG 32

## (13) INFORMATION FOR SEQ ID NO. 12:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 31  
(B) TYPE: Nucleic acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE:  
(A) Description: Other nucleic acid
- (iii) HYPOTHETICAL: No
- (iv) ANTISENSE: No
- (vi) ORIGINAL SOURCE:  
(B) STRAIN:



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